ISOLATION AND CHARACTERIZATION OF IMPURITIES OF CEPHALOSPORINS

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CERTIFICATE

This is to certify that the thesis entitled "ISOLATION AND CHARACTERIZATION OF IMPURITIES OF CEPHALOSPORINS," submitted in fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY (ANALYTICAL CHEMISTRY) is a record of bonafide research carried out by ANURAG TRIVEDI at Lupin Research Center, Mandideep and Lupin Research Park, Pune, under our supervision and the manuscript is suitable for submission for the award of degree of DOCTOR OF PHILOSOPHY IN ANALYTICAL CHEMISTRY.

This is to further certify that ANURAG TRIVEDI has put in minimum 200 days attendance in the Department of Analytical Chemistry during the course of this study.

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CEPHALOSPORINS

Introduction

Cephalosporin C is the parent substance from which the first cephalosporin to find clinical use were derived. Cephalosporin C is produced in very small amount by a wild strain of a species of Cephalosporium [Commenwealth Mycological Institute Kew (C.M.I.) NO. 49137], similar to Cephalosporium acremonium, which was isolated by Brotzu. Mutants of this strain have been obatined that produce Cephalosporin C in much higher yield. Certain strains of Emericellopsis terricola var. glabra may also produce small quantities of this antibiotic.[1]

In addition to Cephalosporin C, the Cephalosporium sp. C.M.I. 49137 produces Cephalosporin N, which is related chemically to Cephalosporin C, and an entirely different antibiotic, Cephalosporin P.[2,3,4] Cephalosporin N, now known as Penicillin N, is identical with an antibiotic which was formerly named synnematin B.[5] Work that began with the study of the later product has shown that the ability to produce Penicillin N is shared by a number of different fungi.

In 1943 certain members of the Fusarium-Cephalosporium group were found by Waksman and Horning to be antagonistic to the growth of bacteria on a solid medium. Eight years later Gottshall et al[6] reported that an antibiotic was produced by a member of the genus Tilachlidium

and by Cephalosporium charticola. The Tilachlidium was then found to be a new species of Cephalosporium and it was named C. salmosynnematum.[7] The name Synnematin, later changed to Synnematin B, was thus given to the antibiotic concerned, whose chemical nature was not then known.[8]

The perfect stage of C. salmosynnematum was observed in 1957 by Grosklags and Swift and this organism was then classified as a new species of the genus Emericellopsis Van Beyma (E. salmosynnematum). Several species of Emericelloipsis were shown to produce Penicillin N[9,10] (synnematin), and the later was isolated from the culture fluid of C. Chrysogenum Thirum and Sukapure.[11,12] Only Cephalosporin P was found in culture fluids of E. humicola, and none of these species were repoted to produce Cephalosporin C. Penicillin N has also been reported to be formed by a member of the genus streptomyces[13] and by Paecilomyces persicinus.[14] However, the configuration of the α-aminoadipic acid residue in the products from these organisms does not appear to have been determined.

It has been suggested by Mangallam et al.[15] that the organism isolated by Brotzu should be classified as a strain of C. chrysogenum and by Gams[16] that it should be described as a strain of Acremonium chrysogenum.

Development

After the demonstration at Oxford of the chemotherapeutic properties of penicillin, a search for antibiotic producing organisms were made by Giluseppe Brotzu in Sardinia. Brotzu began this work in July of 1945, and has examined the microbial flora of seawater near a sewage outlet at Cagliari, supposing that the process of self-purification of the water might be due in part to bacterial antagonism. From a spot that is now reclaimed land he isolated a fungus which he concluded was similar to C. acremonium. When grown on agar this organism secreted material that inhibited the growth of a variety of gram-positive and gram-negative bacteria. Selection of colonies from hundreds of serial cultures on agar plates led to the isolation of a strain that produced significant amounts of antibiotic material when grown in glucose-starch broth. From the filtrates of such cultures a crude active concentrate was obtained after precipitation of inactive products with ethanol.

Both culture filtrates and crude active concentrates from the Cephalosporium sp. were tested clinically in Sardinia. The filtrates were injected directly in to staphylococcal and streptococcal lesions, particularly boil and abscesses, with results that were reported to be good. The concentrates were given intravenously and intramuscularly to patients with typhoid fever, paratyphoid A and B infections, and brucellosis. Although the treatment was complicated by pain and by

pyrogenic effects, it usually appeared to produce marked improvement particularly in cases of typhoid fever. Brotzu believed that his results offered hopeful prospects, but he concluded that isolation of the active principle would be beyond his resources and expressed the hope, at the end of his publication, that the work would be taken up elsewhere.

The initial experiments at oxford with the Sardinian Cephalosporium sp. (C.M.I. 49137) were carried out by N.G. Heatley, who found that culture fluids contained an acidic antibiotic which was readily extractable into organic solvents. After July 1949 the production of culture fluids was carried out and at the same time the study of the active material was undertaken by H.S. Burton and E.P. Abraham. Attention was first concentrated on the antibiotic extractable into organic solvents, but it became clear that this substance, which was called Cephalosporin P because it showed activity only against certain gram-positive bacteria, was not the antibiotic described by Brotzu.

In August 1949 a second antibiotic was found in Oxford to be present in the culture fluid of the Cephalosporium sp. This substance remained in the aqueous phase after the extraction of Cephalosporin P and was discovered independently at Clevedon. It was active against gram-negative as well as gram- positive bacteria and was named Cephalosporin N.[17,2] There appeared to be little doubt that Cephalosporin N was responsible for the antibacterial activity that had first been observed 4 years earlier in Sardinia.

The first clear evidence that Cephalosporin N was a new type of Penicillin was obtained early in 1952, when E.P. Abraham and G.G.F. Newton showed that a partially purified sample of the antibiotic yielded the characteristic amino acid Penicillamineine (β,β-dimethylcysteine) on acid hydrolysis.

At this stage it was decided to increase the effort to isolate and characterize Cephalosporin N. It was found that the culture medium became deficient in methionine during the fermentation. Addition to the medium of methionine, in particular the D isomer, was then shown to increase the yield of Cephalosporin N,[18] and following the resulting improvement in supply, this antibiotic was finally isolated by Abraham et al.[19] in a form that was nearly pure.

Cephalosporin N yielded D-α-aminoadipic acid as well as penicillamine on acid hydrolysis.[3] Further studies left no doubt that it had the structure (Figure-1) with a residue of D-α-aminoadipic acid linked through its δ-carboxyl group to the nucleus of the penicillin molecule.[20] It was subsequently renamed Penicillin N.

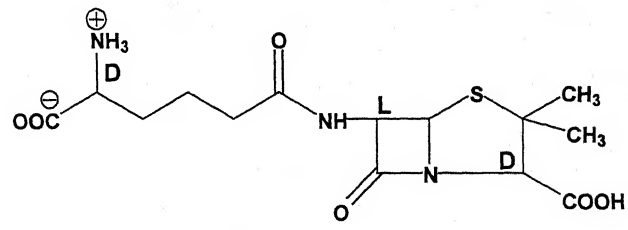

Figure-1

Chemical Studies

The prospects of obtaining Cephalosporin C in relatively large amounts were much improved when a search for higher yielding mutant strains of the Cephalosporium sp., undertaken in 1957 by B.K. Kelly and his colleagues at the Antibiotics Research Station, Clevedon, began to be rewarding. Mutant 8650, which produced much more Cephalosporin C than wild strain, was used in subsequent fermentations. This eventually led to a supply of antibiotic from Clevedon, which was sufficient for its chemical structure to be determined.

Progress in studies on the chemical degradation of Cephalosporin C enabled a structure to be proposed for this substance at Oxford, in April 1959, which was later confirmed by further chemical work[21] and by an X-ray crystallographic analysis.[22] Attention was then turned by Newton and Abraham with Bronwen Loder, to the possibility of obtaining the nucleus of the Cephalosporin C molecule, stimulated by a report published by Batchelor et al.,[23] of the characterization and isolation in quantity of the penicillin Nucleus, 6-aminopenicillanic acid. Since Cephalosporin C had the same D-α-aminoadipyl side chain as the penicillinase sensitive Penicillin N, it appeared that its resistance to staphylococcal penicillinase depended on its ring system, and a comparison of the antibacterial activities of Penicillin N and benzylpenicillin suggested that appropriate changes in the side chain of Cephalosporin C would lead to compounds with much higher

activities, at least against gram-positive bacteria. The relative stability of Cephalosporin C ring system enabled the later to be obtained in very low yield, by mild acid hydrolysis of Cephalosporin C. A small amount of this substance, 7-aminocephalosporanic acid, had been isolated in a relatively pure state and its N-phenylacetyl derivative had been shown to be much more active than Cephalosporin C against a penicillinase-producing strain of S. aureus.[24] However, the problem of producing 7-aminocephalosporanic acid in quantity remained to be solved.

Several pharmaceutical companies expressed interest in the cephalosporins at a relatively early stage. The Distillers Company (Biochemicals) Ltd. made contact with the Oxford workers through Sir Howard Florey in 1954 and considered the possibility of providing a supply of Penicillin N. In 1955 Imperial Chemical (Pharmaceuticals) Ltd. initiated a personal connection with the object of becoming acquainted with the current research on Penicillin N and Cephalosporin C. In the same year an informal suggestion came to Oxford from Eli Lilly and Company that a liaison might be arranged for the purpose of producing Cephalosporin C. Glaxo also showed serious interest.

In 1956 N.R.D.C. began to organize meetings between members of Glaxo's staff and research workers at Oxford and Clevedon. The difficulty of producing Cephalosporin C in any quantity from low-yielding Cephalosporium sp. Slowed the pace of further development.

However, after the higher - yielding mutant 8650 had become available in 1957, 100gm of Cephalosporin C was ultimately produced in the Glaxo Laboratories and some of this material was used in the last experiments made to confirm the chemical structure which had already been proposed.

In 1958 A.M. Van-Arendonk, director of Eli Lilly patent division, approached the National Research Development Corporation and discussed with B.J.A. Bard and J.C. Cain a proposal for a program aimed at the production of substances related to Cephalosporin C, including the nucleus of the molecule, by fermentation. This idea stemmed from work by E.H. Flynn and his colleagues, which was then under way, on the isolation of the Penicillin Nucleus, 6-aminopenicillanic acid. Eli Lilly signed an agreement with the Corporation in January 1959. Although the project concerned was not successful, they entered into a general option agreement early in 1960 under which they received mutant 8650 of the Cephalosporium sp. and access to technical information. From then on they were to make increasingly important contributions to the cephalosporin field.

Other pharmaceutical companies were now showing interest in the cephalosporins. A general option for a license had been obtained from N.R.D.C. by E.R. Squibb and Company in 1959. In addition to Eli Lilly, three U.S. Companies, Merck and Company, Chas. Pfizer and company, and Smith Kline and French, entered into option agreements

in the following year, as did CIBA in Switzerland and Farmitalia in Italy. In 1961 a similar agreement was made with the Fujisawa Pharmaceutical Company in Japan. Until this time it had appeared possible that Cephalosporin C itself might find some clinical use for treatment of penicillin-resistant staphylococcal infections, even though its very low specific activity would presumably have required it to be given by intravenous infusion. But this appeared unlikely after the production of 2, 6-dimethoxyphenylpenicillin (methicillin) from 6-aminopenicillanic acid and the demonstration of its Chemotherapeutic properties.[25,26] Thus, a great deal depend on the discovery of a method for the production of 7-aminocephalosporanic acid on a large scale.

Extensive searches in several laboratories for an enzyme which would remove the D - α - aminoadipyl side chain from Cephalosporin C had no significant success. But before the end of 1960 an ingenious chemical procedure had been discovered in the Lilly Research Laboratories, which enabled the side chain to be removed, and 7-aminocephalosporanic acid to be obtained in very much higher yield than was possible by simple hydrolysis.[27] Detailed of an improved version of the original process were reported to the N.R.D.C. By this time work at Eli Lilly and at Glaxo had opened the way to the production of Cephalosporin C in large amount by fermentation. Thus, 7-amonocephalosporanic acid became available in quantity and an intensive study of the properties of derivatives of the compound soon

led to the introduction of two cephalosporins, namely cephalothin and cephalorodine, into medicine.

In comparison with that of many other antibiotics the first stages of history of cephalosporins were unusual and prolonged. Eight years elapsed between the isolation of the Cephalosporium sp. in Sardinia and the discovery of Cephalosporin C in Oxford, largely because the activity of this substance in a conventional assay. A further 7 years passed before the isolation of high-yielding mutant strains of the organism and the discovery of a novel method for obtaining the nucleus of the molecule allowed the potentialities of the new ring system of the latter to be adequately explored. During this latter period the difficulties to be overcome appeared at a times to be so formidable that it would not have been surprising if the project had been abandoned. Its final success must be attributed to a combination of scientific ability, technical expertise and willingness to take calculated risk in the pharmaceutical companies that were mainly involved.

COMPARISON OF PENICILLINS AND CEPHALOSPORINS

While a number of Penicillanic acids are produced in fermentations of Penicillium fungi, β-lactam antibiotics from other microorganisms are obtained as derivatives of α-aminoadipic acid. Although these compounds have not been of clinical use per se, comparison of

Cephalosporin C and Penicillin N has historical significance. Chemical modification of these naturally occurring substances has provided compounds which indicate the types of structural variation capable of producing more potent antibiotics. One example of this, already noted, is the conversion of Cephalosporin C to Cephalosporin C_A by reaction with pyridine. More recently, several streptomycetes have been shown to produce β-lactam antibiotics closely related to those produced by fungi. The first reported instance of β-lactam production by a streptomycete was the isolation of Penicillin N in the Merck Laboratories.[28] Other β-lactams originating from streptomyces species are those reported by Nagarajan et al.[29] They include a cephalosporin having a C-3 carbamoyloxymethyl moiety, another which has a methoxyl in place of hydrogen at C-7, and a third having both of the foregoing substituents in the same molecule.

The antibiotic activity of β-lactam antibiotics, which occur in nature as compared to cephalothin. Several derivatives prepared by reaction carried out at the 3-methyl position are included to indicate trends observed in early work on these compounds by Abraham.[30] Both Penicillin N and Cephalosporin C have less than 1% of the activity of benzylpenicillin against a penicillin-sensitive staphylococcus,[31] but they show superior activity against gram-negative organisms, with Penicillin N being more potent in several cases and Cephalosporin C in others. The penicillin is more active against a penicillin-sensitive S. aureus

(3055) where 1.0 mg/ml solutions of each produced zone diameters of 18 and 14 mm, respectively. The replacement of the acetoxyl function (Cephalosporin C) on the 3-methyl group by a primary carbamate produces little change in in-vitro antibacterial activity. Within the limits of the test these compounds appear equivalent. In the α-aminoadipic acid- containing antibiotics the presence of a methoxyl function at C-7 results in diminished activity against the gram-positive microorganisms. In contrast, a significant increase in inhibitory properties toward gram-negative bacteria is observed. The qualitative gram-negative bacterial spectrum of these methoxy-substituted antibiotics is almost the same as for Cephalosporin C.

The reaction of 7-ACA derivatives with nucleophiles has been studied extensively, and many examples of these products have been cited. The reaction of Cephalosporin C with pyridine was reported by Hale et al.[31] In contrast to most variations of structure, the resulting pyridinium betaine shows enhancement of both gram-negative and gram-positive activity.

A more common pattern observed when looking at new β-lactam antibiotic derivatives is a shift in antibacterial spectrum. For example, the 7-methoxyl Cephalosporin C derivatives show a decrease in activity toward gram-positive organisms along with the increase in activity against the gram-negative bacteria. This shift in antimicrobial spectrum appears to be consistent with correlation mentioned earlier

concerning the differences in lipophilic character of the cell walls of various bacteria.

These changes in biological spectrum of the α-aminoadipoyl derivatives are strongly suggestive of effects observed also with other 7-amido functions and have therefore been of great predictive value in structure-activity studies.

One may generalize that in non β-lactamase producing gram-positive bacteria the penicillin will be more inhibitory. Chauvette and co-workers commented on this fact very early while working with cephalosporins which, when directly compared in Oxford units of activity, were only about a fifth as active as the corresponding penicillin. They found that structural requirements for high activity were similar for side-chain amides of cephem and penam antibiotics. The difference in relative levels of inhibition by penam as compared with the cephem[32] derivatives. Available evidence, cited earlier, attributes this intrinsic difference to a greater chemical reactivity of the β-lactam in the penicillin ring system.

The situation is more complex when comparing the activity of penicillanic and cephalosporanic acid toward gram-negative bacteria. The occurrence of β-lactamases is more common and their specificity has been shown to be more divers in gram-negative organisms.[33] A penicillin β-lactamase since inhibition by the penicillins listed is uniformly poorer than for the cephalosporins, while less differences is

evident when E.coli N10 is considered. Against many gram-negative organisms the MIC values are equivalent. Rarely, however, does the penicillin exhibit a superior level of activity. Serratia marcescens (X99) is one of these exceptions and generally responds to penicillins at lower MIC values than to cephalosporins, i.e. a penicillin (ampicillin) MIC of 8.8 μg/ml and a cephaloglycin MIC of 68 μg/ml are found for X99.

Naito and co-workers have compared several derivatives of sydnone-3-acetamido substituted penicillins and cephalosporins and find a broad spectrum of biological activity in both the penam and cephem compounds. Again, the penicillins are somewhat more active against gram-positive bacteria and less active against the gram-negative organisms used. Stedman et al. Investigated a series of pyridylacetamidocephalosporanic and penicillanic acid which also conformed to these generalizations. However, Raap and Micetich found consistently higher activity for a series of substituted isothiazolylmethylpenicillins over the corresponding cephalosporins using strains of E.coli and several Salmonella. MIC values were determined with a twofold serial dilution technique.

The effects of penicillin β-lactamase-resistant penicillins are evident from the dramatic drop in MIC values with the resistant staphylococcus organisms cited when they are exposed to methicillin or oxacillin. Price states that these compounds have only 2 and 10 % respectively of the

activity of the penicillin G against sensitive staphylococci. S. aureus X400 is resistant to all penicillins including methicillin. This strain whose resistance is not well understood is also resistant to most cephalosporin antibiotics.

The beta-lactam family of antibiotics includes many of the most heavily used antibacterials in clinical medicine. They are important, both historically and currently, because of their effectiveness and generally low toxicity. The beta-lactam structure is being exploited by many drug development groups in the search for new drugs with improved efficacy against resistant strains of bacteria.

The majority of the clinically useful beta-lactams belong to either the penicillin (penam) or cephalosporin (cephem) group. Moxalactam is an antibacterial that belongs to the oxacephem group, which is closely related to the cephalosporins. For convenience, it is common to include it as a member of the cephalosporins because its pharmacology is so closely related to the so-called third generation cephalosporins. The beta-lactams also include the carbapenems (e.g. imipenem), the monobactams, e.g. aztreonam, and the beta-lactamase inhibitors, (e.g. clavulanic acid).

Cephalosporins are semisynthetic antibiotic derivatives of Cephalosporin C, a substance product by the fungus Cephalosporium acremonium. All commercially available cephalosporins contain the 7-

aminocephalosporinic acid (7-ACA) nucleus which is composed of a β-lactam ring fused with a 6-membered dihydrothaizine ring. Addition of various groups at R1 (position 7) and R2 (position 3) of Cephalosporin Nucleus results in derivatives with differences in spectra of activity, stability against hydrolysis by β̃ lactamases, protein binding, GI absorption, or susceptibility to desacetylation.

Currently available cephalosporins are generally divided into 4 groups based on spectra of activity:

- **First generation cephalosporin**
- **Second generation cephalosporin**
- **Third generation cephalosporin and**
- **Fourth generation cephalosporin**

First generation cephalosporins

First generation cephalosporins usually are active in vitro against gram-positive cocci including penicillinase producing and nonpenicillinase producing Staphylococcus spp. First generation cephalosporins have limited activity against gram-negative bacteria that they are inactive against enterococci (e. g. Enterococcus faccalis), methicillin- resistant staphylococci, Bacteroides fragilis, Citrobactor, Listeria monocytogenes, Proteus other than P. mirabilis, Providencia,

Pseudomonas and Serratia. Example Cefadroxial, Cefazolin, Cephalexin, Cephapirin, Cephradine and Cephalothin.

Second-generation cephalosporins

Second generation cephalosporins usually are active in vitro against bacteria susceptible to first generation drugs are active in vitro against most strains of Haemophilus influenzae concluding ampicillin-resistant strains) and more active in vitro against gram-negative bacteria than first generation cephalosporins. Example Cefaclor, Cefamandole, Cefmetazole, Ceforanide, Cefotetan, Cefoxitin, Cefprozil, Cefuroxime axetil and Loracarbef.

Third generation cephalosporins

Third generation cephalosporins usually are less active in vitro against susceptible staphylococci than first generation cephalosporins; however, the third generation drugs have an expanded spectrum of activity against gram-negative bacteria compared with the first and second generation drugs which are also active in-vitro against Citrobacter, Enterobacter, E.coli, Klebsiella, Proteus, Morganella, Providencia and Serratia that may be resistant to first and second generation cephalosporins. Some parenteral third generation drugs

have activity in-vitro against B. fragilis and Pseudomonas. Third generation cephalosporins are inactive against most slaphylococci, enterococci (E. faccalis) and L. monocytogenes. Example: Cefdinir, Cefixime, Cefoperazone, Cefotaxime, Cefpodoxime, Ceftizoxime, Ceftriaxone, Ceftazidime and Ceftibuten.

Fourth generation cephalosporins

Fourth generation cephalosporins, alike third generation, have an expanded spectrum of activity against gram-negative bacteria compared with the first and second-generation drugs. However, fourth generation cepharosporins are active in-vitro against some gram-negative including Pseudomonas aeruginosa and certain Enterobacteriaceae that generally are resistant to third generation cephalosporins. In addition, fourth generation cephalosporins may be more active against gram-positive bacteria than some third generation drugs. The extended spectrum of activity of this generation cephalosporins is related to the fact that the drug penetrates the outer cephalosporins and the fact that the drug is more resistant inactivation by chromosomally and plasmid-mediated β-lactamase than most other cephalosporins. Example Cefepime, Cefpirome.

Mechanism of Action

A summary of the mechanism of action of the beta-lactam antibacterial should be stated as follows. β-lactam antibiotics inhibits bacterial cell wall synthesis. The drugs cause nicks in the peptidoglycan net of the cell wall that allow the bacterial protoplasm to "flow" from its protective net into the surrounding hypotonic medium. Fluid accumulates in the naked protoplast, as the cell now devoid of its wall is called, and it bursts resulting in death of the organism.

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DISCUSSION

Introduction

The control of impurities is currently a critical issue to the pharmaceutical industry. The International Conference on Harmonization (ICH) has formulated a workable guideline regarding the control of impurities. Herein I have discussed in depth the different types and origins of impurities, in relation to ICH guidelines, including degradents and their formation pathway with specific examples.

Impurities in pharmaceuticals are the unwanted chemicals that remain with the active pharmaceutical ingredients (APIs), or develop during formulation, or upon aging of both API and drug products. The presence of these unwanted chemicals even in small amounts may influence the efficacy and safety of the pharmaceutical products. Impurity profiling (i.e., the identity as well as the quantity of impurity in the pharmaceuticals), is now getting receiving important critical attention from regulatory authorities. The different pharmacopoeias, such as the European Pharmacopoeia (EP), British Pharmacopoeia (BP) and the United States Pharmacopoeia (USP), are incorporating limits to allowable levels of impurities present in the APIs or formulations. The International Conference on Harmonization (ICH) has published guidelines on impurities in new drug substances,[1]

products,[2] and residual solvents.[3] There is a good significant demand for the impurity reference standards along with and the API reference standards for both regulatory authorities and pharmaceutical companies. According to ICH guidelines on impurities in new drug products, identification of impurities below than 0.1% level is not considered to be necessary unless the potential impurities are expected to be unusually potent or toxic. In all cases, impurities should be qualified. If data are not available to qualify the proposed specification level of an impurity, studies to obtain such data may be needed (when the usual qualification threshold limits given below are exceeded). According to ICH, the maximum daily dose qualification threshold is considered as follows:

≤ 2g/day 0. 1 % or 1 mg per day intake (whichever is lower)

≥ 2g/day 0.05%

Sources of Impurities in Medicines

Medicines are the formulated forms of active pharmaceutical ingredients. There are 2 types of impurities in medicines: -

1. Impurities associated in with active pharmaceutical ingredients
2. Impurities that form are created during formulation and or with aging or that are related to the formulated forms.

Impurities associated in with APIs

According to ICH guidelines, impurities associated with APIs are classified into the following categories:

- Organic impurities (Process and Drug-related)
- Inorganic impurities
- Residual solvents

Organic impurities

Organic impurities may arise during the manufacturing process and/or storage of the drug substance. They may be identified or unidentified, volatile or non-volatile, and include the following:

- Starting materials or intermediates
- By-products
- Degradation products
- Reagents, ligands, and catalysts
- Enantiomeric impurities

Starting materials or intermediates

These are the most common impurities found in every API unless a proper care is taken in every step involved in throughout the multi-step synthesis. Although the end products are always washed with solvents, there are always chances of having the residual unreacted starting materials may remain unless the manufacturers are very careful about the impurities. In Ceftiofur Sodium bulk, there is a limit test for thiol which could be a starting material for one manufacturer or be an intermediate for another.

By-products

In synthetic organic chemistry, getting a single end product with 100% yield is very rare; there is always a chance of having by-products. In the case of Cefprozil API Cefprozil carbonate (Figure 1) may form as a by-product.

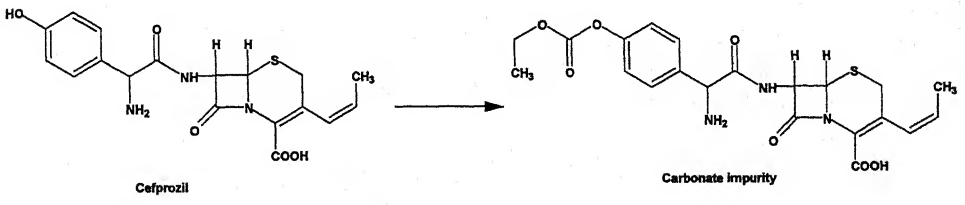

Figure 1.

Degradation products

Impurities can also be formed by degradation of the end product during manufacturing of bulk drugs. However, degradation products resulting from storage or formulation to different dosage forms or aging are common impurities in the medicines. The degradation of cephalosporins is a well-known example of degradation impurities in the products. The presence of a β-lactam ring as well as that of an amino group in the C7 side chain plays a critical role in their degradation.[4] (Figure-2)

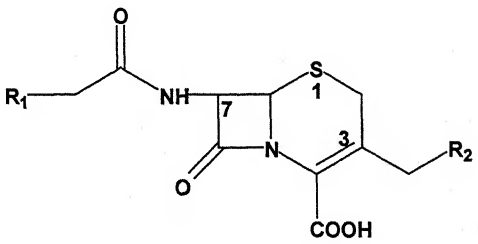

Figure 2 - General Structures of Cephalosporins.

Reagents, ligands, and catalysts

These chemicals are less commonly found in APIs; however, in some cases they may pose a problem as impurities.

In general, an individual API may contain all of the above-mentioned types of organic impurities at levels varying from negligible to significant.

The residual solvents associated with these APIs have also been determined.[5]

As the organic impurities are the most common product as well as process related impurities, it is the responsibility of both the APIs manufacturers and the users (i.e., formulators) to take care of these impurities according to ICH guidelines or compendia.

In addition, for an optically active single isomer drug there could be enantiomeric impurities present in the API.

Enantiomeric impurities

The single enantiomeric form of a chiral drug is now considered as an improved chemical entity that may offer a better pharmacological profile and an increased therapeutic index with a more favorable adverse reaction profile.[6]

Inorganic impurities

Inorganic impurities may also derive from the manufacturing processes used for bulk drugs. They are normally known and identified and include the following:

- Reagents, ligands, and catalysts
- Heavy metals
- Other materials (e.g., filter aids, charcoal)

Reagents, ligands, and catalysts

The chances of having these impurities are rare, however, in some processes, these could create a problem unless the manufacturers take proper care during production.

Heavy metals

The main sources of heavy metals are the water used in the processes and the reactors (if stainless steel reactors are used), where acidification or acid hydrolysis takes place. These impurities of heavy metals can easily be avoided using demineralized water and glass-lined reactors.

Other materials (e. g. filter aids, charcoal)

The filters or filtering aids such as centrifuge bags are routinely used in the bulk drugs manufacturing plants and, in many cases, activated carbon is also used. The regular monitoring of fibers and black particles in the bulk drugs is essential to avoid these contaminations.

Residual solvents

Residual solvents are organic volatile chemicals used during the manufacturing process or generated during the production. It is very difficult to remove these solvents completely by the work-up process however, efforts should be taken to the extent possible to meet the safety data. Some solvents that are known to cause toxicity, should be

avoided in the production of bulk drugs. Depending on the possible risk to human health, residual solvents are divided into 3 classes.[3] Solvents such as benzene (Class I, 2 ppm limit) and carbon tetrachloride (Class I, 4 ppm limit) are to be avoided. On the other hand, the most commonly used solvents such as methylene chloride (600 ppm), methanol (3000 ppm), pyridine (200 ppm), toluene (890 ppm), N,N-dimethylformamide (880 ppm), and acetonitrile (410 ppm) are of Class II. Class III solvents (acetic acid, acetone, isopropyl alcohol, butanol, ethanol, and ethyl acetate) have permitted daily exposures of 50 mg or less per day. In this regard, ICH guidelines[3] for limits should be strictly followed.

Impurities related to formulation

Apart from bulk drug-related impurities, the formulated form of API may contain impurities that form in various ways.

Impurity forms during formulation

a) Method related

The impurities like Cefotaxime lactone in Ceftiofur and Delta isomer of Cefuroxime Axetil (Figure-3) is formed during process. The formation of these impurities has been found to depend on the pH of the reaction.

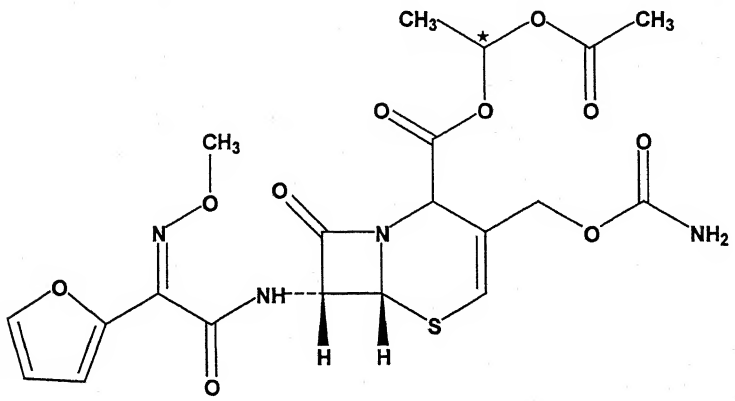

Figure- 3

b) Environmental related

The primary environmental factors that can reduce stability include the following:

- Exposures to adverse temperatures

There are many APIs that are labile to heat or tropical temperatures. For example, Cephalosporins as drug substances are very heat-

sensitive and degradation frequently leads to loss of potency, especially in liquid formulations.

- Light-especially UV light

Several studies have reported that cephalosporins are unstable under tropical conditions such as light and heat, for e.g. If ceftiofur was exposed in sun light its E- isomer is increased substantially.

- Humidity

For hygroscopic products, humidity is considered detrimental to both bulk powder and formulated solid dosage forms.

c) Dosage form factors related

Although the pharmaceutical companies perform pre-formulation studies, including a stability study, before marketing the products, sometimes the dosage form factors that influence drug stability force the company to recall the product. In general, liquid dosage forms are very much susceptible to both degradation and microbiological contamination. In this regard, water content, pH of the solution/suspension, compatibility of anions and cations, mutual interactions of ingredients, and the primary container are critical factors.

Microbiological growth resulting from the growth of bacteria, fungi and yeast in a humid and warm environment may result in oral liquid products that are unusable for human consumption. Microbial

contaminations may occur during the shelf life and subsequent consumer-use of a multiple-dose product due to inappropriate use of certain preservatives in the preparations[7] or because of the semi-permeable nature of primary containers.

Formation of impurities on aging

a) Mutual interaction amongst ingredients

Most cephalosporins are very labile and on aging they pose a problem of instability in different dosage forms, especially in liquid dosage forms.

b) Functional group- related typical degradation

- Hydrolysis

Hydrolysis is a common phenomenon for the ester type of drugs. Examples include cefuroxime axetil, cefpodoxime proxetil.

- Oxidative degradation

In the presence of atmospheric oxygen, cephalosporins may form sulfoxide. Example Ceftiofur.

- Photolytic cleavage

Pharmaceutical products are exposed to light while being manufactured as a solid or solution, packaged, held in pharmacy shops or hospitals pending use, or held by the consumer pending use. Mostly cephalosporins are very labile to photo-oxidation. In susceptible

compounds, photochemical energy creates free radical intermediates, which can perpetuate chain reactions. Most compounds will degrade as solutions when exposed to high energy UV exposure. Cetiofur acid gets converted in to its E- isomer when exposed to sunlight. (Figure-4)

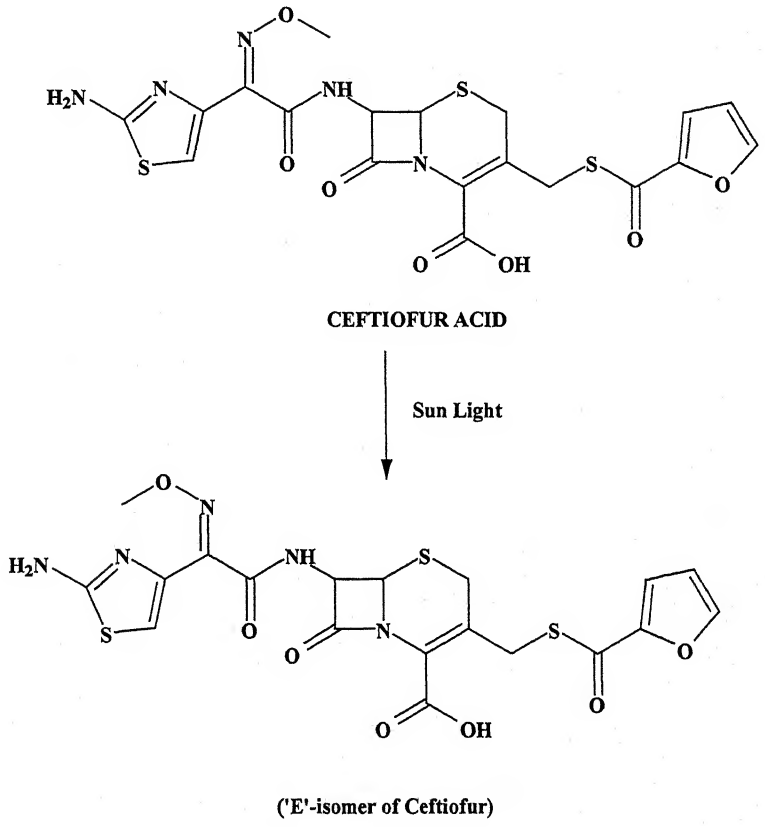

Figure -4

- Decarboxylation

Some cephalosporins, such as cefdinir, lose carbon dioxide from the carboxyl group when heated in acedic condition.

The identification of degradation products can provide an understanding of impurity formation and define degradation

mechanisms. If the identification process is performed at an early stage of drug development, there is adequate time for improvements in the drug substance process and drug product formulation to prevent these impurities and degradants long before the filing stage. Impurity and degradant structure elucidation is a collaborative effort involving the analytical chemist, process chemist and/or formulator as well as the degradation, mass spectrometry and NMR experts.

The process of identification of impurities and/or degradants begins early in drug development. The first step of the process is to determine at what level the unknown is present. According to the ICH Guidelines on Impurities in New Drug Substances, the studies conducted to characterize the structure of actual impurities present in the new drug substance at a level greater than 0.1% (depending on the daily dose calculated using the response factor of the drug substance) should be described. It is to be noted that all specified impurities at a level greater than the identification threshold in batches manufactured by the proposed commercial process should be identified. Degradation products observed in stability studies at recommended storage conditions should be similarly identified. When the identification of an impurity is not feasible, a summary of the laboratory studies demonstrating the unsuccessful effort should be included in the application.

According to the ICH Guidelines on Impurities in New Drug substance degradation products observed in stability studies conducted at recommended storage conditions should be identified when present at a level greater than the identification thresholds (1% for a maximum daily dose of <1 mg to 0.1% for a maximum daily dose of > 2 g). Identification of impurities below the 0.1% level is generally not considered to be necessary unless the potential impurities are expected to be unusually potent or toxic. Therefore, it is imperative to determine the level of the unknown impurity and/or degradant early in the process. If the unknown is below the 0.1% threshold, then a discussion will need to take place among the project team members in order to determine if isolation and identification are necessary. However, if the unknown is at or above the 0.1% limit, then effort should be put forth for identification.

Once a decision has been made to identify an unknown, the next logical step is to evaluate all known process related impurities, precursors, intermediates, and degradation products. By observing the relative retention times (HPLC) of all known process related impurities, precursors and intermediates (if available), one can quickly determine whether or not the impurity of interest is truly unknown. If the relative retention time of the unknown impurity matches that of a standard, then the unknown can be identified using HPLC with ultra-violet (UV) photodiode array as well as mass spectrometry (MS) detection. The

identity is confirmed by correlating the retention time, UV spectra and mass spectra of the unknown impurity with that of the standard.

Identifying an unknown by using a standard, as described in the above paragraph, is a quick and easy process, when the relative retention time of an unknown does not match that of a standard than molecular mass and fragmentation data via HPLC-MS will be required. It is essential to determine the molecular mass of the unknown. Not only does the molecular mass help in the identification of the unknown, but it also enables one to track the correct peak by HPLC if isolation becomes necessary. In order to run LC-MS, a mass spectrometry compatible HPLC method must be available. The mobile phase should contain volatile buffers that are HPLC-MS-compatible.

If the mass spectrometry data evaluation yields sufficient structural information, this eliminates the need to isolate the impurity in question. If standards of the proposed structures are available, they can be correlated with the unknown as previously described. If standards are not available, isolation is required.

An alternative to isolation is small-scale synthesis. If possible structures have been proposed from the mass spectrometry data, one can study the process chemistry and determine at which step of the process the impurity and/or degradant is most likely to be formed. By knowing the process chemistry, the feasibility of the proposed structures can be evaluated. Proposed structures can then be

synthesized if a reasonable synthesis is available. It is easier to synthesize and identify the unknown if the chemistry works quickly (i.e. one step/straight-forward chemistry). If small-scale synthesis is chosen, the synthesis must be the most efficient route.

At this stage of the process, it is frequently necessary to isolate and characterize the unknown. One of the most important factors to consider when approaching an isolation experiment is the sample origin. It is vital to determine whether the unknown is an impurity and/or degradant, and to locate a sample that contains an enriched quantity of the unknown. Isolating low level impurities can prove to be very cumbersome and time consuming. Therefore, the ultimate goal is to find a sample that contains an enriched quantity of the unknown. Two great resources of enriched samples are retained mother liquor samples and purposeful degradation/stability samples. If the unknown is a drug substance degradant, then the degradation reaction can be scaled-up to generate a large quantity of the unknown. If it is a drug product degradant, then effort should be put forth to form the degradant in the drug substance so that extraction from the excipients is not required. Whenever enriched samples are not available, the unknown must be isolated from the bulk drug substance or drug product.

A number of methods can be used for isolating impurities and/or degradants. Three of the most utilized techniques are thin-layer

chromatography (Preparative TLC), flash chromatography (column chromatography), and preparative high performance liquid chromatography (HPLC). The actual technique used depends upon the nature of the impurity and/or degradant, including the amount present in the original material from which it must be isolated. A good starting point is to assess the separation that is currently being used by the analytical chemist. Does the current methodology provide optimum resolution of the impurity/degradant from the main band and other impurities, and if so, is that method by TLC or HPLC? This is a key factor in determining which technique to utilize. Each of the three techniques will be discussed separately.

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ISOALTION TECHNIQUES

Thin-Layer Chromatography

Preparative thin-Layer Chromatography (TLC) is a good technique to use when normal phase solvents provide optimum separation. Typical thin-layer separations are performed on glass plates that are coated with a thin layer of stationary phase. The stationary phases used in TLC encompass all modes of chromatography including adsorption, normal and reverse phase, ion exchange, and size-exclusion chromatography. The equipment required is simple and inexpensive. It is an ideal technique for the isolation of compounds because of its simplicity. However, in order for TLC to be successful, the impurity and/or degradant should be at or above the 1% level. Anything below this level is very difficult to isolate on a TLC plate due to higher detection limits.

The steps involved in preparative TLC are:

- Application of the sample onto the plates
- Development of the plates
- Detection and location of the compound of interest
- Extraction of the compound of interest.

Detection is usually by ultraviolet light. When the separated compound of interest is located on the plate, the band is scraped and the impurity is extracted from the stationary phase (i.e. silica gel) with an

appropriate solvent. The extracted material is filtered or centrifuged, and the solvent collected is evaporated to yield the isolated material. It is essential to remove silica gel and other interferences that may inhibit the identification of the compound. The isolated material is then submitted for LC-MS analysis.

One of the main disadvantages of using TLC for preparative isolations is that limited amounts of material can be isolated from the plates. Using preparative TLC plates can avoid this problem. Preparative plates contain thicker films of stationary phase, thus allowing larger amounts of sample to be applied. Even in cases where preparative plates are used, chances are good that not enough material can be isolated to obtain traditional NMR analysis (including 1H and 13C NMR). Therefore, TLC is most useful when an impurity and/or degradant is identifiable by LC-MS. In cases where NMR analysis is essential for identification, flash chromatography and/or preparative HPLC are more suitable techniques.

Flash Chromatography

(Column chromatography)

When an existing normal phase TLC method provides adequate resolution of the impurity/degradant to be isolated, then flash chromatography can be a useful technique. Flash chromatography is a simple absorption chromatography technique for the routine

purification of organic compounds. It allows for separations of samples weighing 0.01-10.0 g in 10 to 15 minutes. Flash chromatography is a rapid, inexpensive and easily performed technique with a large sample capacity (approximately 5 times the load of reverse phase packing materials). Normal phase flash chromatography is ideal when the sample is soluble in non-polar or moderately polar solvents such as hexane, chloroform, and dichloromethane. These volatile solvents allow easier concentration of impurities and degradants. Before choosing flash chromatography as the separation technique, chemistry knowledge to assess the potential stability of the isolated product prior to isolation in order to determine if special collection conditions are necessary, is required. For example, collect thermally unstable products in chilled flasks.

The first step in developing a flash chromatographic separation is to determine the optimum solvent composition by analytical TLC. A solvent system is chosen that provides good resolution and moves the desired impurity and/or degradant to Rf = 0.354. TLC can provide a guide to suitable solvent systems, but development work may be needed in order to optimize the separation when it is scaled-up to the larger I.D. columns used in flash chromatography. A suitable column is selected based upon the resolution of the impurity and/or degradant and the amount of sample to be purified. The amount of sample that can be purified on a column is dependent upon the resolution of the

impurity and/or degradant, and it is proportional to the cross sectional area. If less resolution is required to separate a key degradant or impurity, sample load can be significantly increased. Typically, each run on a flash column can chromatograph gram quantities of material depending on the column size. The column load is typically much higher than that of reverse phase chromatography.

The columns are generally packed with silica gel. In order for the separation to be successful, the size of the silica gel should be 40-63 mm4. A concentrated solution of the sample is prepared. The sample solution is applied at the top of the column, and the walls of the column are washed with a few milliliters of eluent. Solvent is added to the column, and air pressure is applied at a flow rate of 2 inches/minute to rapidly elute the desired impurity and/or degradant. Separation is based upon the differential interactions between the solute molecules and the adsorbent surface of the silica gel. Fractions are continuously collected and monitored by chromatographic techniques (HPLC with UV detection, GC, or TLC). The fractions containing the compound of interest are combined and evaporated to dryness. The isolated material is cleaned (post-isolation cleanup, such as small scale column or analytical HPLC re-injection, is essential) and submitted for LC-MS and NMR analysis.

Flash chromatography is a fast and inexpensive technique for isolations requiring only moderate resolution. Typically, compounds

having Rf > 0.15 can be cleanly separated using this technique, and separations at Rf @ 0.10 are possible. In cases where high resolution is required, flash chromatography can be used as a preliminary purification step. It can be a good method of concentrating and partially purifying complex mixtures, thus making the final isolation much easier by preparative HPLC. For example, the main band can be isolated from the rest of the mixture; therefore, the impurity mixture needing optimum resolution can be injected with higher load on HPLC. In addition, automated flash chromatographic systems with UV detectors and fraction collectors are available that further simplify the isolation process.

Preparative HPLC

Preparative chromatography is the process of using liquid chromatography to isolate a sufficient amount of pure unknown compound(s) for the purpose of structure elucidation by spectroscopic techniques, which are often referred to as semi-preparative. The scale of preparative HPLC is normally larger than that of conventional HPLC. Therefore, a practical starting point is to develop an analytical separation that optimizes the isolation conditions. Optimization of the analytical method implies seeking conditions which combine maximum resolution of the peak of interest and minimum

elution time, under the restriction of a limited pressure drop. The optimized conditions determine the column, mobile phase, flow rate and sample loading capacity for the particular column. The conditions may be either normal phase or reverse phase. The mobile phase should be chosen carefully to avoid salt complexation with the compound to be isolated. Volatile acid salts such as trifluoroacetic acid, formic acid and acetic acid are acceptable mobile phase additives, and the ammonium counter-ion is preferred for pH adjustment to any of these acids.

Once the analytical scale method conditions are optimized, the next step is to choose a column and scale-up the analytical HPLC parameters so that preparative chromatography can be performed and the unknown compound(s) isolated for identification by MS and NMR. For ease of transition, a preparative column consisting of the same packing material and particle size should be chosen. The column is the most important component of the process. The column determines the amount of material that can be loaded onto the column for the desired purity and recovery. An important step in the scale-up procedure is determining the maximum load on the analytical column. The maximum analytical load is essential in determining the loading capacity of the preparative column. When an appropriate column is chosen, the analytical isolation can be scaled up.

The scale-up factor is used to predict the loading capacity and flow rate for the preparative column. For example, if a separation optimized on a 4.6 mm x 150 mm column was scaled to a 20 mm x 300 mm column, the scale factor for the sample load would be 38. Thus, the scale-up factor multiplied by the maximum analytical load estimates how much material can be loaded on the preparative column.

To maintain the same resolution when scaling-up a method, the flow rate also needs to be scaled proportionally. The preparative flow rate can be estimated by using the scale-up factor. For this estimation, the scale-up factor is multiplied by the analytical flow rate to estimate the preparative flow rate.

When the preparative method has been optimized, injections are made and the compound of interest is typically collected using a fraction collector. The fractions are pooled together in a collection vessel. The stability of the isolated product should be assessed prior to isolation in order to determine if special collection conditions are required. The isolated product is concentrated using conventional sample concentration techniques (i.e. distillation at normal or reduced pressure, precipitation, freeze-drying, solvent extraction, and membrane filtration). Rotary evaporation and flash distillation are the two most commonly used techniques to recover isolated products from the mobile phase. After the product has been recovered, it should be dried under high vacuum to remove all solvents. An analytical clean up

of the isolated sample is critical prior to NMR analysis. A clean sample improves the purity and quality of NMR data. As was mentioned earlier, volatile acid salts such as trifluoroacetic acid, formic acid and acetic acid are often used as mobile phase additives, which may cause salt formation if the pH of the mobile phase is adjusted (i.e. using ammonium hydroxide). In addition, mobile phase solvents may also contain low-level impurities that become enriched during the concentration process. It is essential to remove any salts and/or impurities from the isolated product. A simple purification method for the isolated product is to re-inject it onto the preparative column using a mobile phase without any additives or pH adjustments. By utilizing gradient elution, salts can be removed by incorporating an aqueous rinse at the beginning of the run, and then the organic solvent can be ramped to elute the desired product. Thus, the isolated peak is purified. Solid-phase extraction also offers great potential in purifying the isolated product because of its universality. Additionally, washing the isolated sample with deuterated solvent several times also helps to prepare the sample for NMR experimentation. Once the sample has been purified, it is submitted for LC-MS and NMR analysis.

As was mentioned earlier, isolation of low-level impurities and/or degradants can be quite labor intensive. Consider a 0.1% level impurity present in a drug substance bulk lot. Based on traditional NMR experiments, 5 mg of the impurity would be needed to obtain

structural confirmation. To isolate 5 mg of the impurity from the bulk, a minimum of 5 g of bulk drug substance would be needed, assuming 100% recovery. Because actual recoveries are generally closer to 50% for low level (0.1% range) isolations, 10 g of bulk drug substance would generally be requested.

In addition to requiring significant bulk material, the timeframe to complete the isolation is considerable. If the maximum analytical load for a 4.6 mm x 150 mm column has been determined to be 5 mg of the parent drug substance, assuming the isolation will be performed using semi-preparative chromatography (20 mm x 300 mm column), and approximately 190 mg of sample can be loaded onto the preparative column. For a 0.1% level unknown, this translates to 190 mg of unknown injected onto the preparative column. Therefore, a total of 27 injections are required. If the assay time were estimated to be one hour, it would take at least 27 hours to perform the injections needed to obtain 5 mg (once again assuming 100 % recovery). This timeframe does not include the time needed for method scale-up development concentration and solubility experiments, as well as mass spectrometry and NMR experimentation.

On the other hand, if a sample was available that contained 10% of the unknown, only 1 gram of bulk would be needed and the estimated timeframe of the isolation would be drastically reduced. In this example above, 19 mg of unknown can be injected onto the preparative column

(assuming the maximum analytical load does not change and resolution is retained with the higher level impurity). Therefore, only one injection would be needed to obtain the amount necessary for NMR analysis, reducing the time to 1 hour.

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RESEARCH WORK

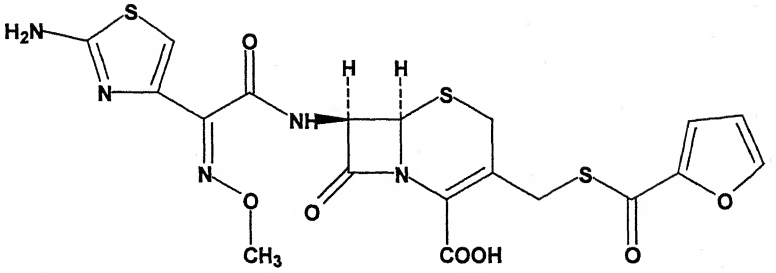
OBJECTIVE

In the light of above discussion it is necessary to produce good quality of active pharmaceutical ingredient (API) as per ICH guide lines, To achieve this, all the impurities present which are more than 0.1% should be isolated and characterized. The efforts would be made to isolate these impurities in pure form using, preparative HPLC system and characterize them with the help of IR, NMR and Mass spectrometer.

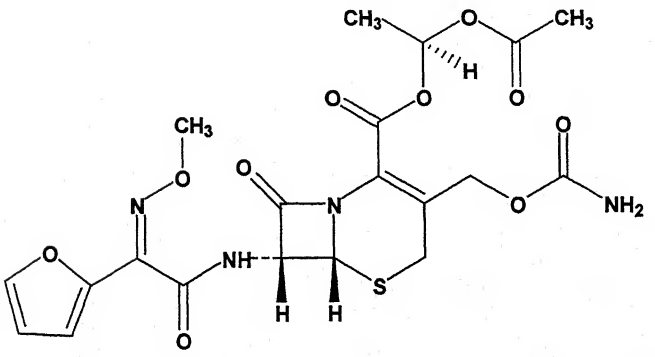
To carry out present research work, Ceftiofur, Cefuroxime Axetil and Cefprozil have been selected for impurity isolation and characterization.

Ceftiofur is a broad-spectrum third generation cephalosporin antibiotics while Cefuroxime Axetil & Cefprozil are second-generation cephalosporin antibiotics, active in vitro against bacteria susceptible to first generation drugs are active in vitro against most strains of Haemophilus influenzae concluding ampicillin-resistant strains and more active in vitro against gram-negative bacteria than first generation cephalosporins.

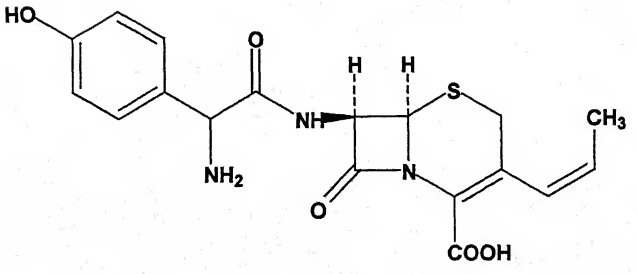
Structures of Ceftiofur, Cefuroxime Axetil and Cefprozil



Ceftiofur



Cefuroxime Axetil



Cefprozil

EXPERIMENTAL SECTION

Materials

Ceftiofur sodium, Cefuroxime Axetil & Cefprozil manufactured in Lupin Ltd, Mandideep (M.P.) was used and all other chemicals used were of reagent grade. Water was purified by milli-Q System.

Instruments

The following instruments were used for the impurity isolation and characterization of Ceftiofur sodium, Cefuroxime Axetil & Cefprozil.

IR spectra were recorded on Perkin-elmer 1650(USA) and Shimadzu FT-IR 8201(Japan) model.

NMR spectra were recorded on Bruker DRX-200 MHz (Switzerland).

Mass spectra were recorded on PE –SCIEX API-3000 (USA) triple quadruple.

pH of solution was measured using Control Dynamic(India).

Balance: Mettler –AE-250 (Switzerland).

Analytical System: Shimadzu binary gradient HPLC equipped with

- System controller: SCL10 ATvp
- Two pumps: LC10ATvp
- Degasser unit: DGU14A
- UV/VIS. Detector: SPD10 Avp
- Autosampler: SIL – 10 ADvp
- Column oven: 10 Asvp
- Data processor: Class-VP –5.03 software
- Column: Inertsil ODS–3V & X-Terra

In-house HPLC methods were developed to separate impurities present in Ceftiofur, Cefuroxime axetil & Cefprozil.

Chromatographic conditions for Ceftiofur

Column

- Type: X-Terra
- Dimensions: 150m x 3.9 mm
- Particle size: 5µm

Detector setting

- Wavelength: 235nm

Mobile phase

- Buffer: 0.01M Ammonium Di hydrogen Orthophosphate + 0.07 % Tetra heptyl ammonium bromide (THAB)
- Mobile A: B — Buffer: Methanol (62:38 v/v)
- pH: 6.5 with dilute Ammonia (10%)
- Flow rate: 1.80ml/min

Chromatographic condition

S.No.	Time	Event	Value	Flow ml/min
1.	0.01	B.conc.	0.00	15.0
2.	5.00	B.conc.	0.00	15.0
3.	10.00	B.conc.	15.00	15.0
4.	20.00	B.conc.	50.00	15.0
5.	25.00	B.conc.	50.00	15.0
6.	30.00	B.conc.	60.00	15.0
7.	35.00	B.conc.	0.000	15.0

Chromatographic conditions for Cefuroxime Axetil

Column

- Type: Zorbax SB Phenyl
- Dimensions: 250 mm x 4.6 mm
- Particle size: 5µm

Detector setting

- Wavelength: 280 nm

Mobile phase

- Buffer: 02.5317 g KH2PO4 + 9.8013 g Tetrabutyl Ammonium Hydrogen Sulphate dissolved in 1400 ml water.
- Mobile A: 775 ml Buffer + 163 ml Acetonitrile + 42 ml THF.
- Mobile B: 600 ml Buffer + 326 ml Acetonitrile + 74 ml THF.
- PH: 2.42 with Phosphoric acid
- Flow rate: 1.30ml/min

Chromatographic condition

S.No.	Time	Event	Value	Flow ml/min
1	0.01	B.conc.	0.00	1.3
2	14.0	B.conc.	0.00	1.3
3	30.0	B.conc.	40.00	1.3
4	55.0	B.conc.	40.00	1.3
5	60.0	B.conc.	0.00	1.3
6	65.0	B.conc.	0.00	1.3

Chromatographic conditions for Cefprozil

Column

- Type: Inertsil C_{18}
- Dimensions: 250mm x 4.6 mm
- Particle size: 5μm

Detector setting

- Wavelength: 280nm

Mobile phase

- Buffer: 0.01 M Sodium Dihydrogen Orthophosphate
- Mobile A: Buffer: ACN (90: 10 v/v)
- Mobile B: Buffer: ACN (30: 70 v/v)
- pH: 4.4 with Orthophosphoric Acid
- Flow rate: 1.5 ml/min

The solutions were filtered and each mobile phase was de-aerated separately.

Chromatographic condition

S.No.	Time	Event	Value	Flow ml/min
1.	0.01	B.conc	0.00	1.5
2.	15.00	B.conc	5.00	1.5
3.	35.00	B.conc	25.00	1.5
4.	70.00	B.conc	50.00	1.5
5.	72.00	B.conc	0.00	1.5
6.	80.00	B.conc	0.00	1.5

Preparative HPLC

The analysis was performed on Preparative HPLC of Shimadzu LC-8A equipped with a system controller SCL-10Avp, auto injector SIL-10AF, UV/Vis detector SPD-10Avp. HPLC data has been processed using class VP-5.03 software.

Preparative isolation

The chromatographic conditions used was as follows:

Column

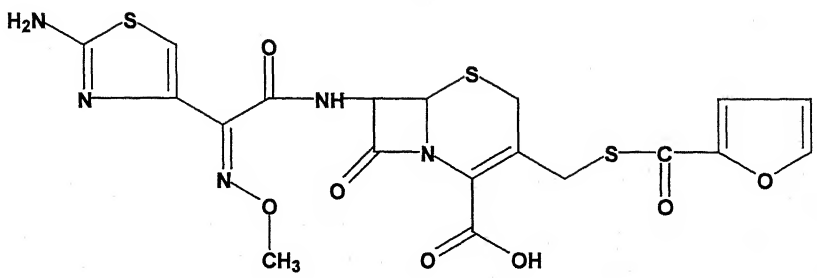
- Column Type: X-Terra C_{18}
- Dimensions: 300 mm x 19 mm
- Particle size: 7 μm

Detector setting

- Wavelength: 235nm
- Flow rate: 15ml/min

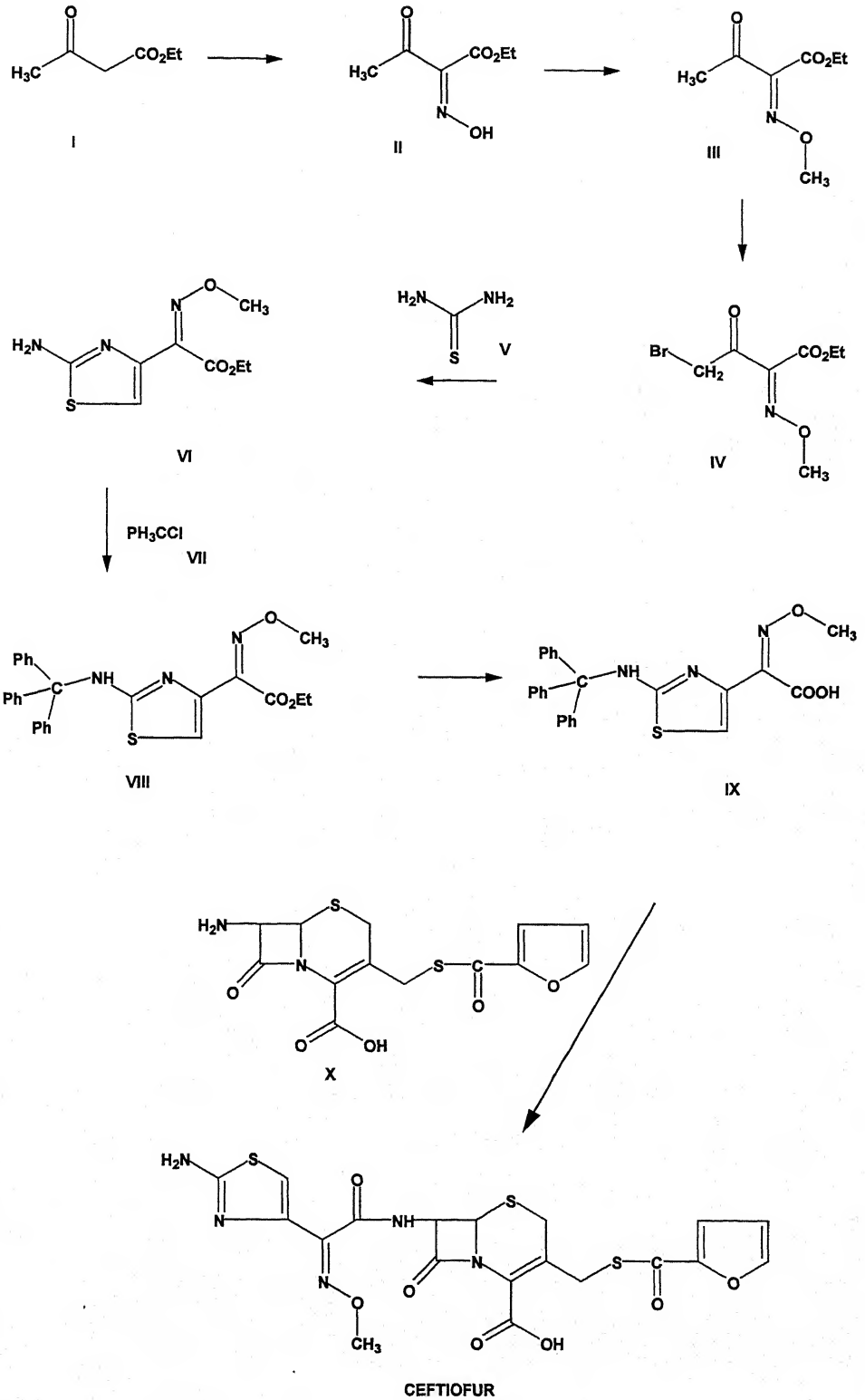
ISOLATION AND CHARACTERIZATION

Product: **Ceftiofur**

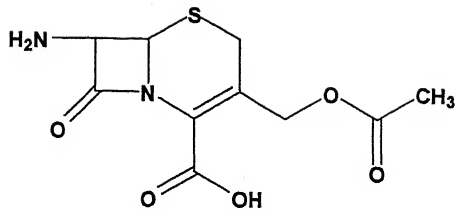


Chemical name: -	[6R-[6alpha,7beta(Z)]]-7-[[2-amino-4-thiazolyl)(methoxyimino)acetyl]amino]-3-[[(2-furanylcarbonyl)thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid
Brand:	Excenel (Upjohn: France) [Vet]
Molecular formula:	$C_{19}H_{17}N_5O_7S_3$
Molecular Weight:	523.57
Uses:	Antibacterial (veterinary)
Chemical Class:	Third generation Beta-lactam antibiotics; Cephalosporin

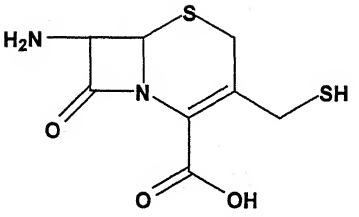
Synthetic Scheme



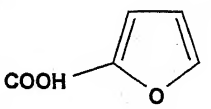
Preparation of X



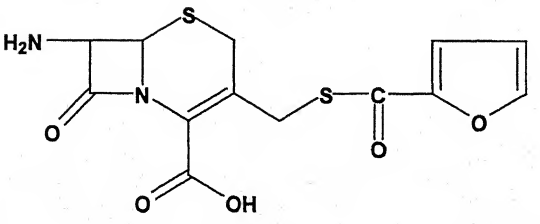
XI



XII



XIII



X

Intermediate used:

I. 3-Oxobutanoic acid ethyl ester

II. 2-(hydroxyimino) acetoacetic acid ethyl ester

III. (Z)-2-(methoxyimino) acetoacetic acid ethyl ester

IV. (Z)-(bromoacetyl) (methoxyimino) acetic acid ethyl ester

V. Thiourea

VI. (Z)-(2-amino-4-thiazolyl) (methoxyimino) acetic acid ethyl ester

VII. Chlorotriphenylmethane

VIII. (Z)-2-(methoxyimino)[2-[(triphenylmethyl) amino]-4-thiazolyl] acetic acid ethyl ester

IX. (Z)-2-(methoxyimino)[2-[(triphenylmethyl) amino]-4-thiazolyl] acetic acid

X. 7-amino-3-[[(2-furanylcarbonyl)thia]methyl]-3-cehem-4-carboxylic acid

XI. 7-ACA

XII. 7-amino-3- (mercaptomethyl)-3-cephem-4-carboxylic acid

XIII. 2-furancarboxylic acid

Reference:

B.I.C 3000 vol. 10 April 2002 Becker Associates Synthesis database.

CHARACTERIZATION OF CEFTIOFUR

1H NMR in DMSO-d6

S.No.	δ ppm	Relative protons	Assignment
1.	3.67-3.33	2	$-SCH_2$
2.	3.95	3	$-OCH_3$
3.	4.31	2	$-CH_2SCO$
4.	5.15	1	6-H
5.	5.79	1	7-H
6.	6.75	1	thiazolyl-H
7.	6.78	1	-Furyl-H
8.	7.25	2	$-NH_2$
9.	7.45	1	-Furyl-H
10.	8.05	1	Furyl-H
11.	9.62	1	-CONH

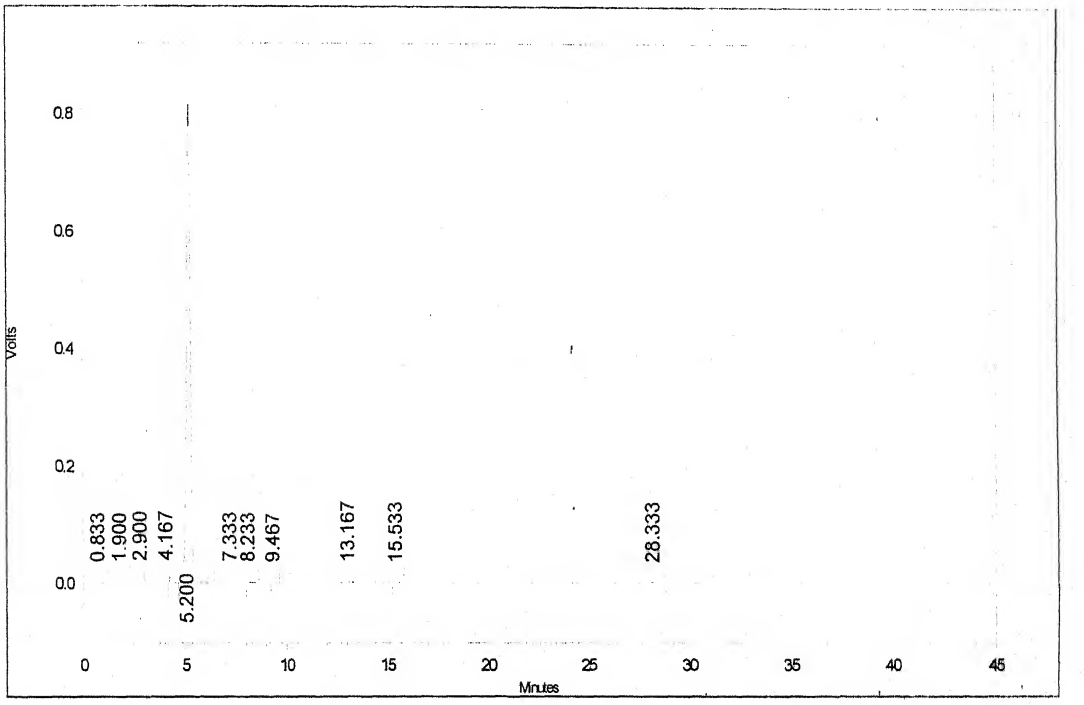
IR SPECTRUM DATA:

cm^{-1}	Functional group
1764.7	-C=O (lactam)
1606.1	-CONH (amide)

MASS SPECTRUM DATA:

Molecular weight	Structure
524	$(M+H)^+$ Molecular ion
546	$(M+Na)^+$

Method Name : E:\HPLC-24\Method\Ceftiofur\Ceftiofur 2.met
File Name : E:\Backup-2002\Data\Nov\Ceftiofur\VU031102.4
Aquired Time : 11/3/2002 6:13:05 PM
Sample ID: Ceftiofur

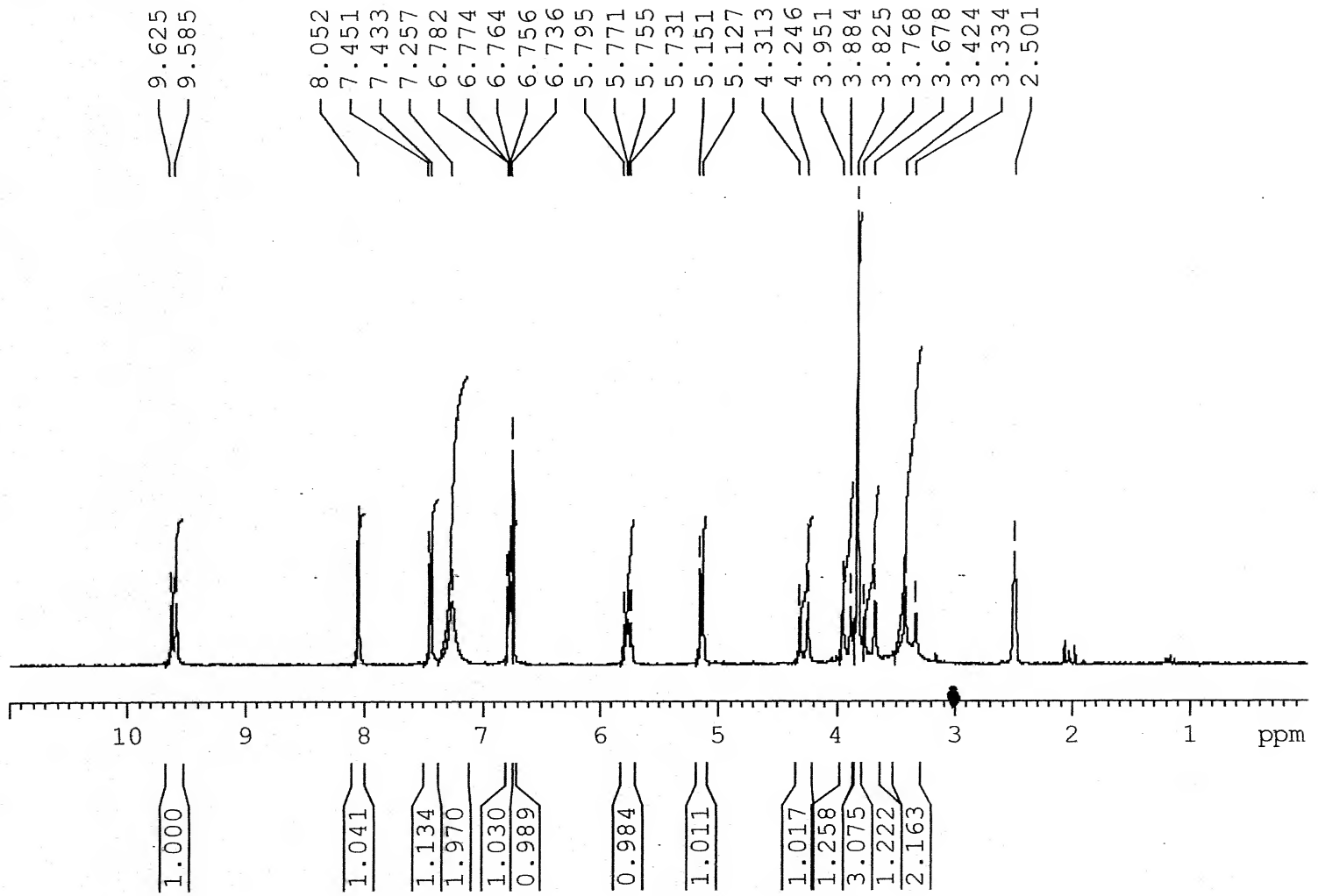


Detector A

Pk #	Retention Time	Area	Area Percent	Relative RT	Name
1	0.833	14296	0.13	0.16	?
2	1.900	14031	0.13	0.37	?
3	2.900	17768	0.17	0.56	?
4	4.167	5520	0.05	0.80	?
5	5.200	10508646	98.96	1.00	Ceftiofur
6	7.333	4564	0.04	1.41	?
7	8.233	18330	0.17	1.58	?
8	9.467	7414	0.07	1.82	?
9	13.167	7102	0.07	2.53	?
10	15.533	4680	0.04	2.99	?
11	28.333	16238	0.15	5.45	?

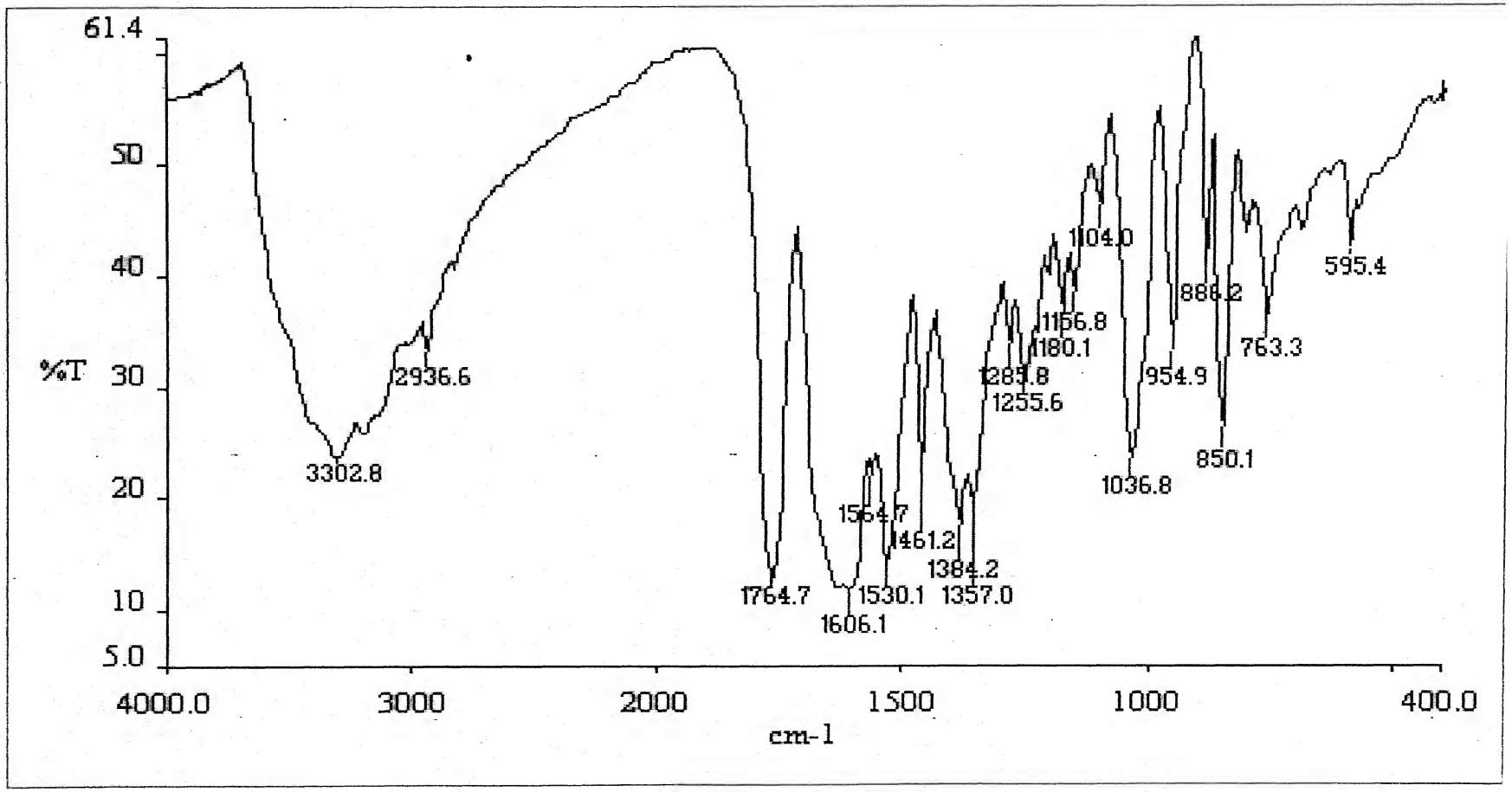
Totals		10618589	100.00		

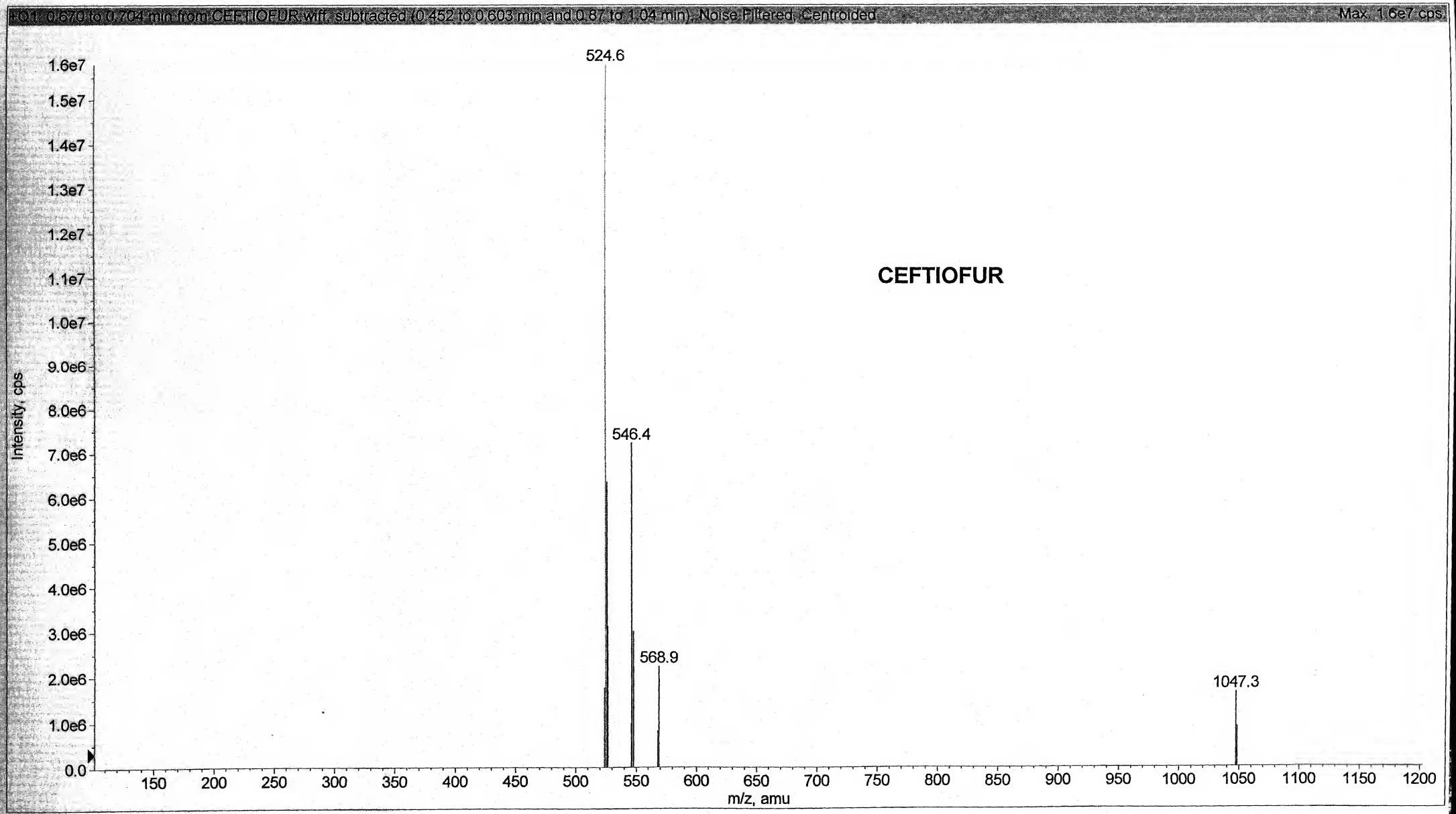
Ceftiofur in DMSO-d6



INSTRUMENT : PERKIN ELMER 1600 Series FTIR

Sample ID : Ceftiofur sodium





99

Sample Name: CEFTIOFUR

OBJECTIVE: - ISOLATION OF IMPURITY AT RRT ~0.16

This impurity at RRT ~ 0.16 was isolated from Ceftiofur sample using preparative HPLC.

Preparative isolation

Column

- Type: X-Terra C_{18}
- Dimensions: 300 mm x 19 mm
- Particle size: 7 µm

Detector setting

- Wavelength: 235 nm
- Flow rate: 15 ml/min

Mobile Phase

- Mobile A: Water (0.1 % Trifluoroacetic Acid)
- Mobile B: Acetonitrile

Sample preparation

~40 mg material dissolved in 4 ml of pH7.0 buffer, filtered and loaded on preparative column

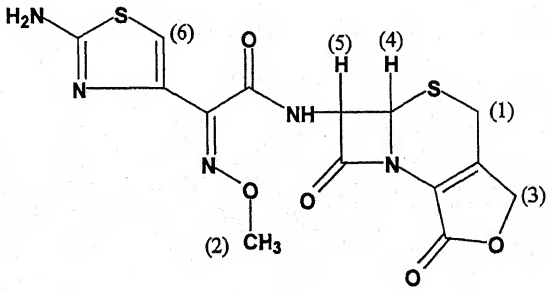
Loading amount: 40 mg sample/injection

Chromatographic condition

S.No.	Time	Event	Value	Flow ml/min
1.	0.01	B.conc	0.00	15.0
2.	5.00	B.conc	0.00	15.0
3.	10.00	B.conc	15.00	15.0
4.	20.00	B.conc	50.00	15.0
5.	25.00	B.conc	50.00	15.0
6.	30.00	B.conc	60.00	15.0
7.	35.00	B.conc	0.000	15.0

Fractions collected were monitored using the analytical method. Fractions having the impurity sample in (~94.0%) purity were concentrated using rotary evaporator at bath temperature 35°C under vacuum to distill out acetonitrile. The combined fractions were then lyophilized to furnish ≥94.0 % (By HPLC area normalization method) pure material, as white solid. The solid obtained was taken for further evaluation of the purity and also for various spectroscopic studies.

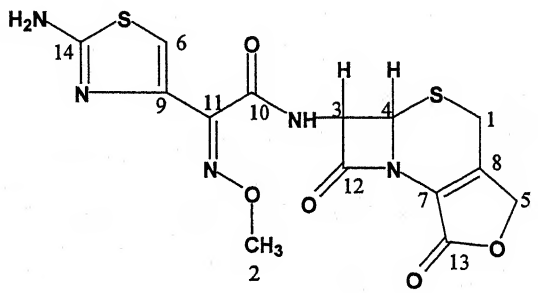
SPECTRAL ANALYSIS



NMR in DMSO-d6

S.No.	Chemical Shift (ppm)	Multiplicity	Relative number of protons	Proton Assignment
1.	9.87	Doublet	1	-NH
2.	6.88	Singlet	1	6
3.	5.97	Doublet	1	5
4.	5.19	Doublet	1	4
5.	5.05	Singlet	2	3
6.	4.02	Singlet	3	2
7.	3.9-3.80	Quartet	2	1

13C NMR



S.No.	Chemical Shift (ppm)	Assignment
1.	170.90	14
2.	167.33	13
3.	162.85	12
4.	161.47	11
5.	144.06	10
6.	141.51	9
7.	133.85	8

S.No.	Chemical Shift (ppm)	Assignment
1.	123.68	7
2.	110.13	6
3.	71.45	5
4.	62.51	4
5.	59.48	3
6.	57.24	2
7.	22.32	1

Analysis of C13 and DEPT 135 Carbon NMR spectrum indicates presence of 14 C atoms and 2 CH2 groups in the molecule.

IR in KBr

The IR spectrum was recorded on FTIR-8201PC Shimadzu instrument using KBr pallet.

S.No.	Frequency Cm^{-1}	Assignment
1.	3387.5-3056.5	-NH, $-NH_2$
2.	2942.9	$-S-CH_2$
3.	1783.0	-C=O Lactam
4.	1746.3	-CO lactone
5.	1652.8	-CONH
6.	1548.0	-CON-
7.	1204.5	γ-Lactone

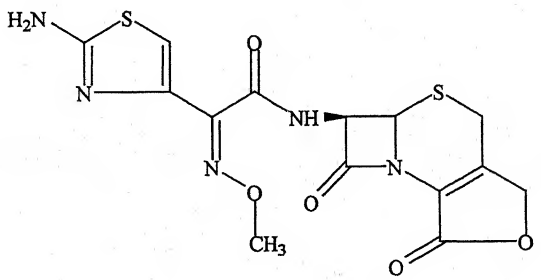
MASS SPECTRA

The compound exhibited a quasi-molecular ion peak at 396 implying a molecular weight of 395. For mass spectrum the sample was dissolved in methanol and spectrum was taken using Atmospheric Pressure Ionization technique having turbolon ionization source. The declustering potential was set at 6 v; focusing potential at 60v, and ionization volt energy (IVE) 4000 at 350°C

Molecular weight	Structure
396.1	$(M+H)^+$ Molecular ion
418.2	$(M+Na)^+$

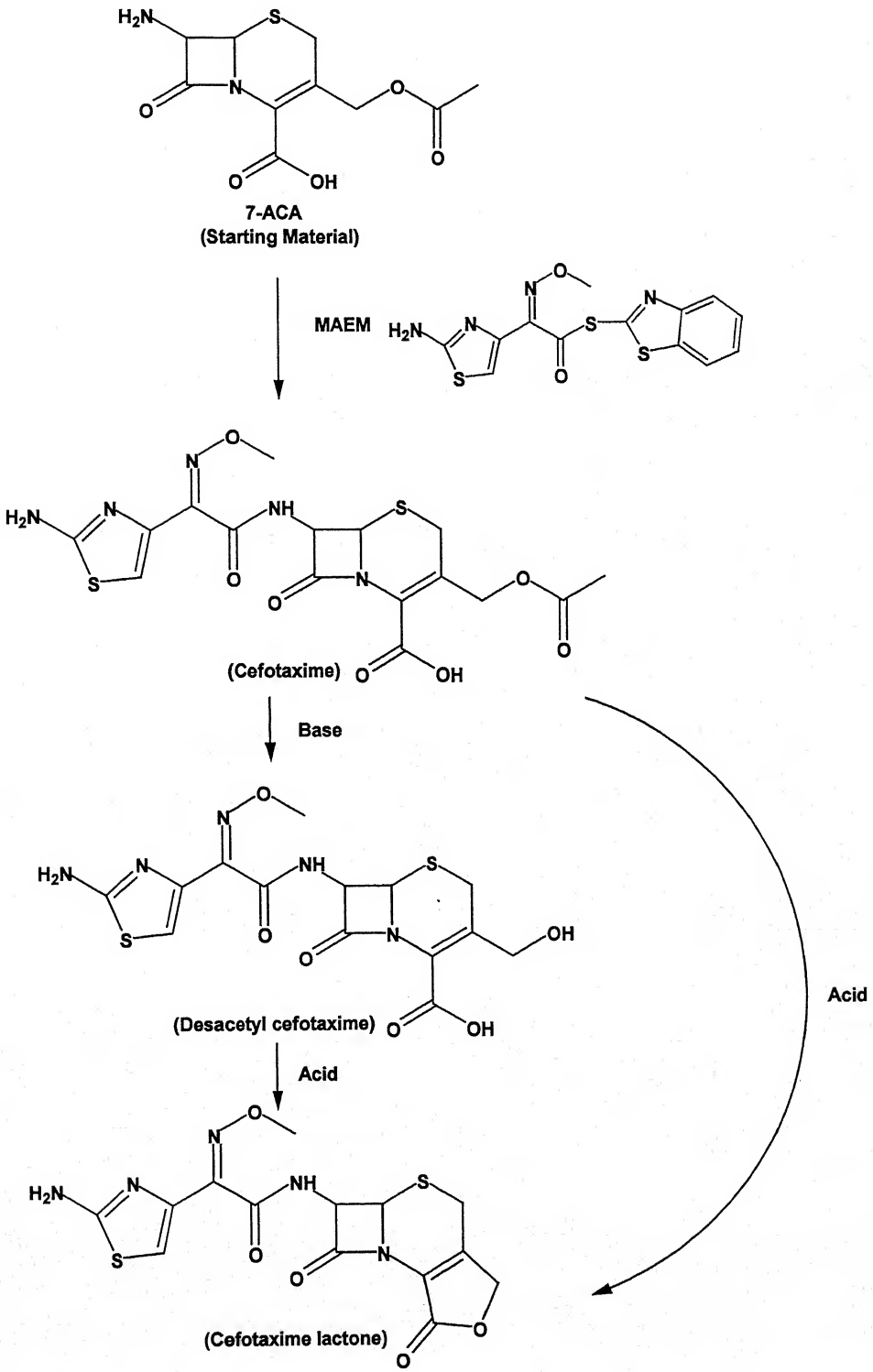
CONCLUSION

The unidentified impurity at RRT 0.16 was isolated from impurity-enriched synthetic sample using preparative HPLC. The structure of this impurity was assigned on the basis of NMR, IR and mass spectra. It was identified as Cefotaxime lactone,

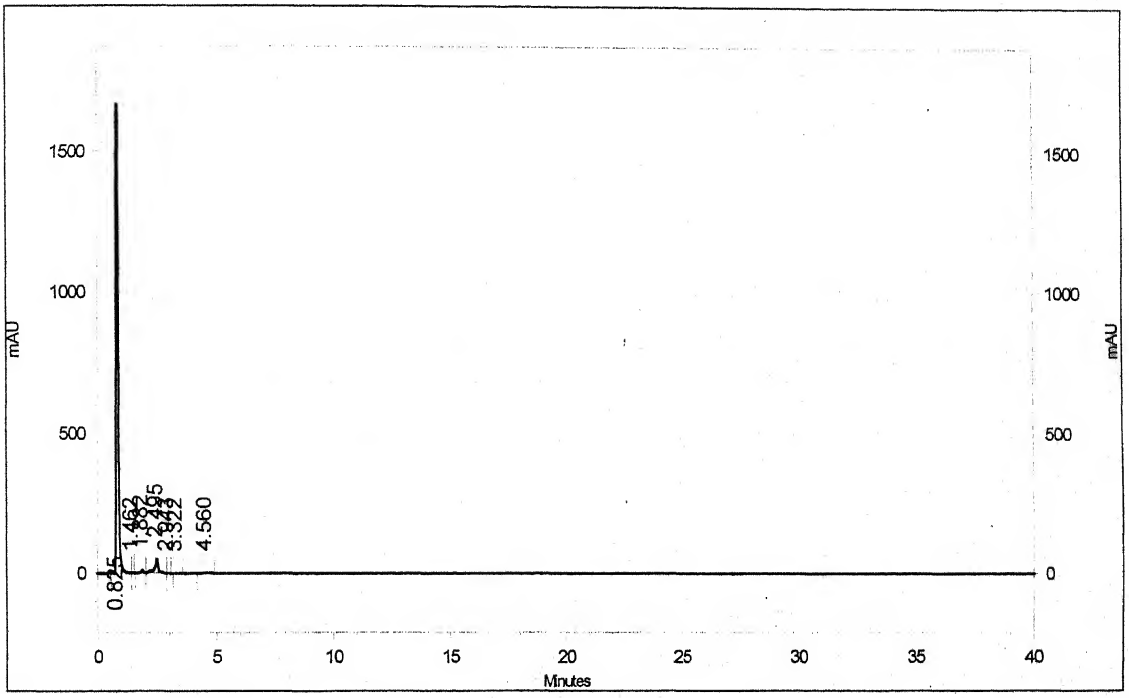


N-((2R)-1,7-dioxo(5-hydro-2H,4H,2aH-azetidino[2,1-b]furano [3,4-d]1,3-thiazin-2-yl))(2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza -3-methoxyprop-2-enamide. (Cefotaxime lactone)

PROPOSED FORMATION PATHWAY OF IMPURITY



Method Name: E:\HPLC-24\Method\Ceftiofur\Ceftiofur 2.met
File Name: E:\HPLC-24\Data\Jan\Ceftiofur\24-012803.4
Aquired Time: 1/28/2003 12:36:31 PM
Sample ID: Cefotaxime Lactone



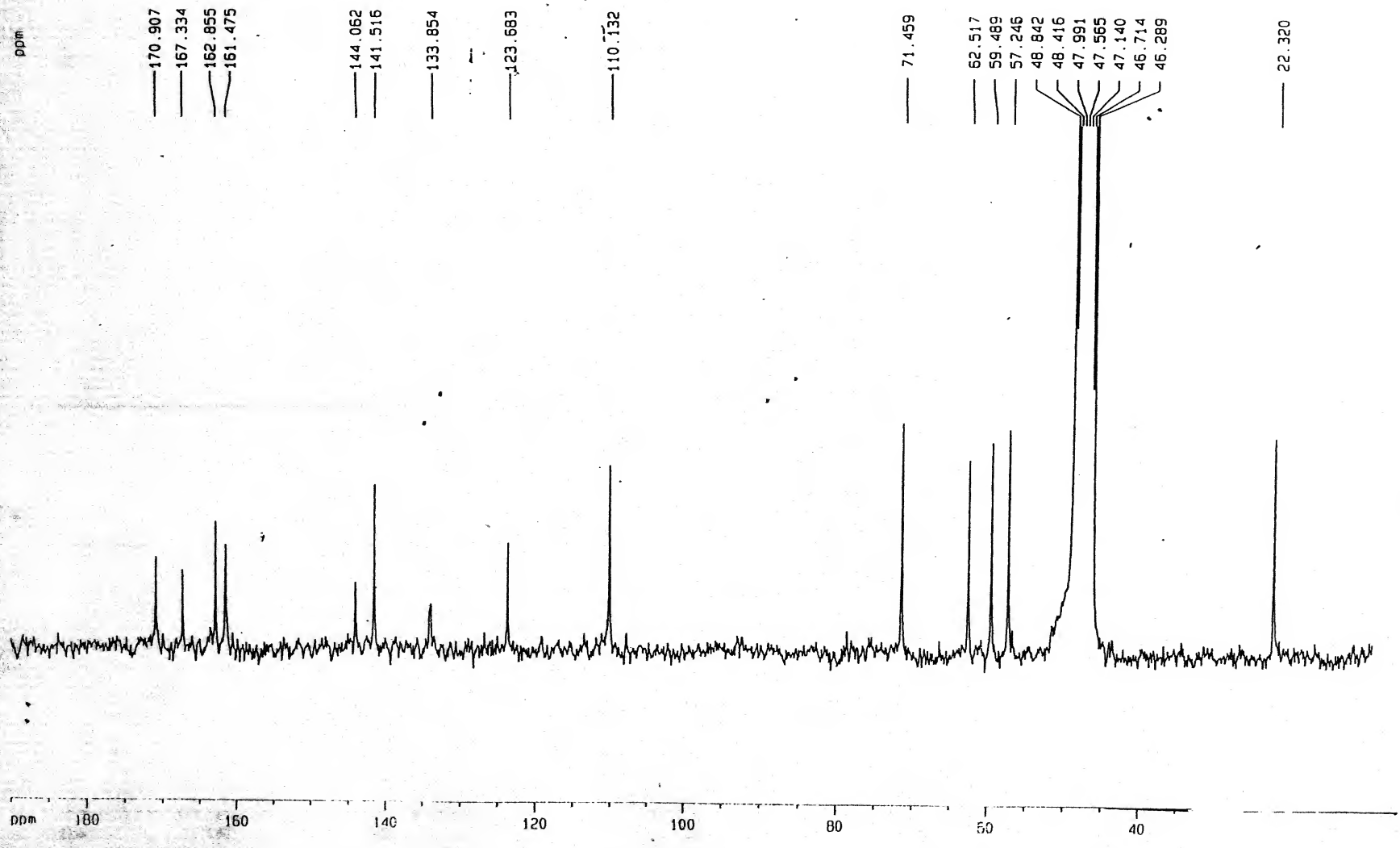
Detector 1-235nm
Results
(Reprocessed)

Pk #	Retention Time	Area	Area Percent	Name
1	0.825	11230107	94.59	**Cefotaxime Lactone**
2	1.462	1217	0.01	
3	1.882	56302	0.47	
4	2.495	525898	4.43	
5	2.943	2877	0.02	
6	3.322	2774	0.02	
7	4.560	53237	0.45	

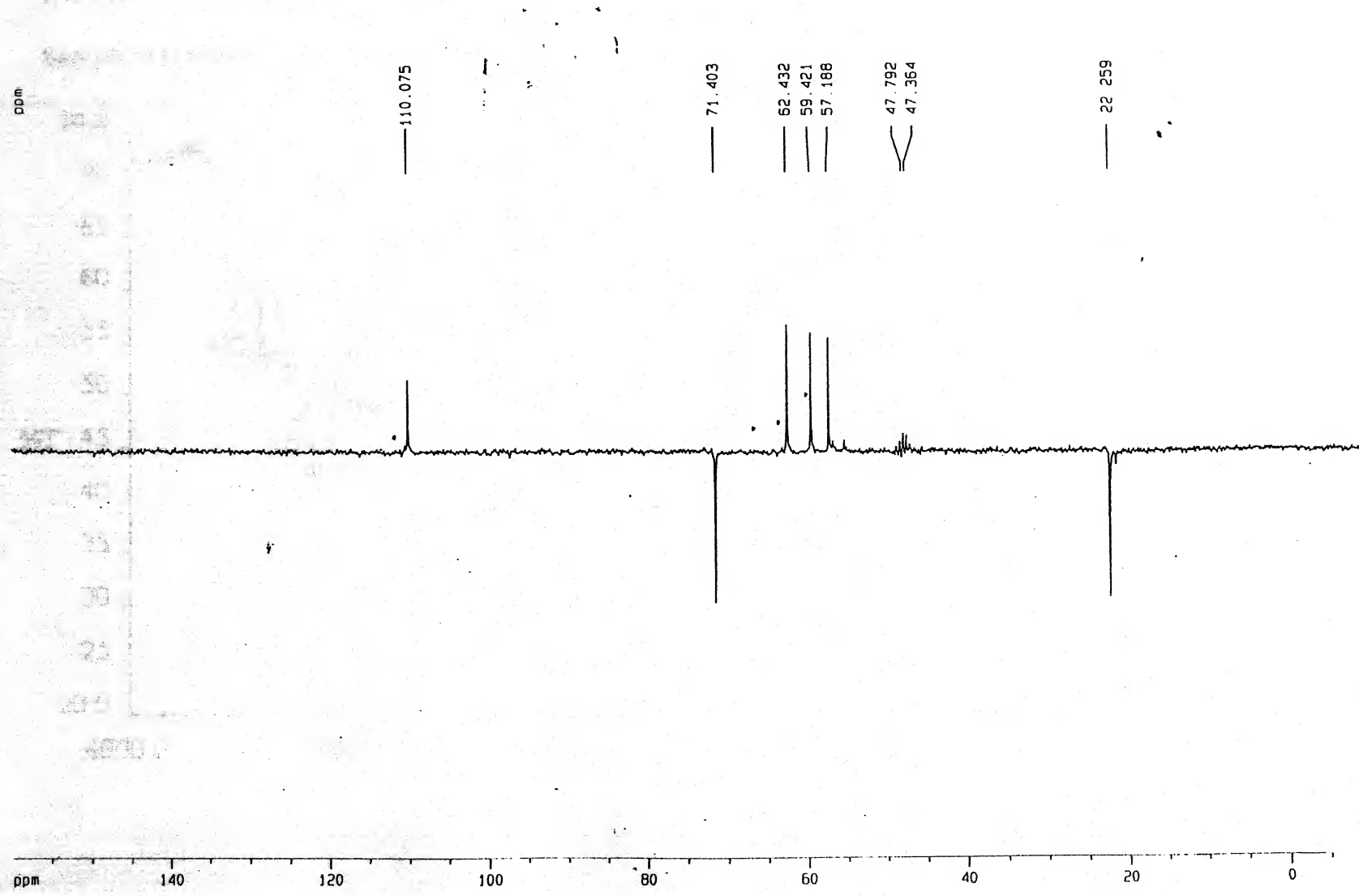
Totals				
	11872412	100.00		

Cefotaxime Lactone in DMSO-d6

^{13}C NMR OF CEFTIOFUR LACTONE.

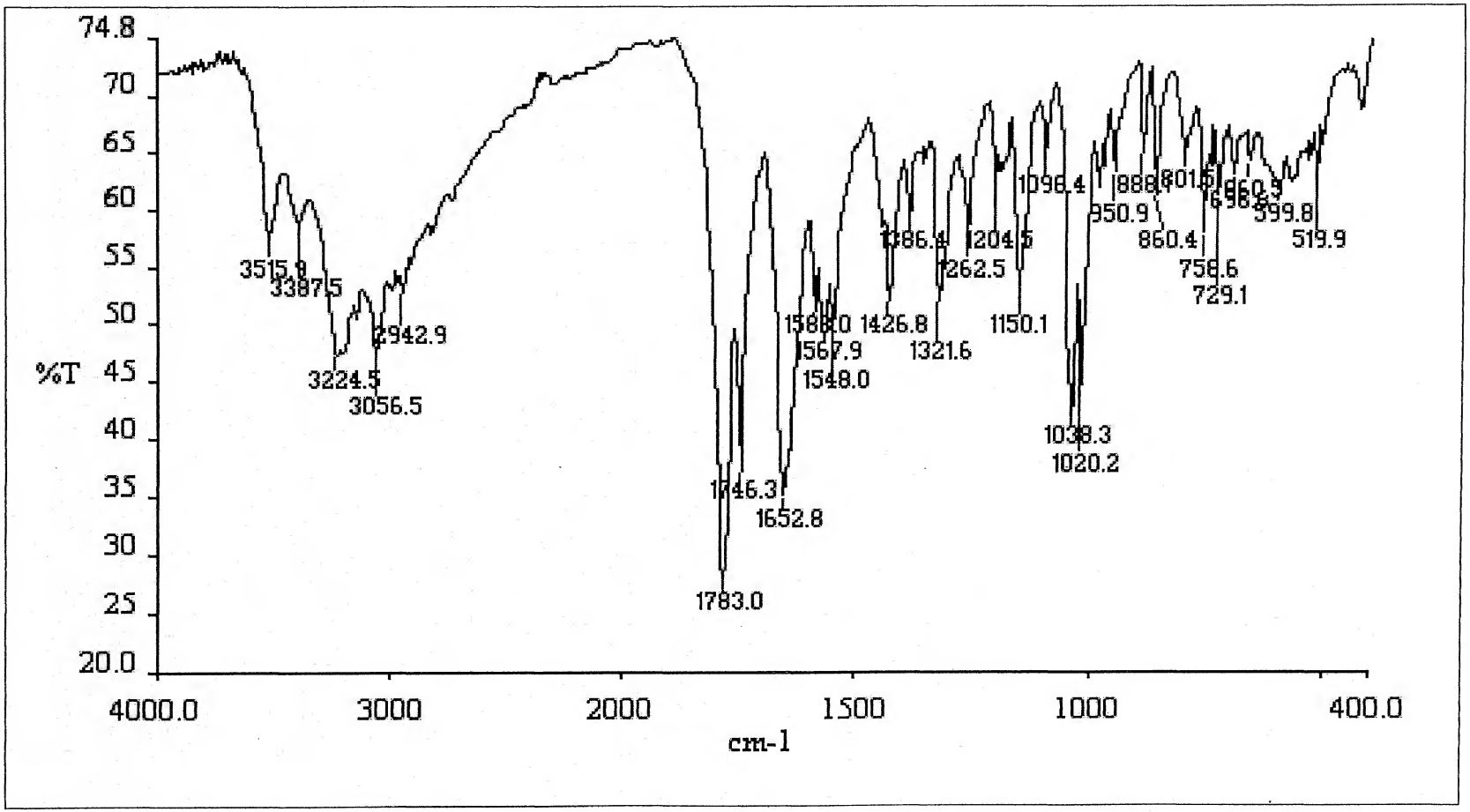


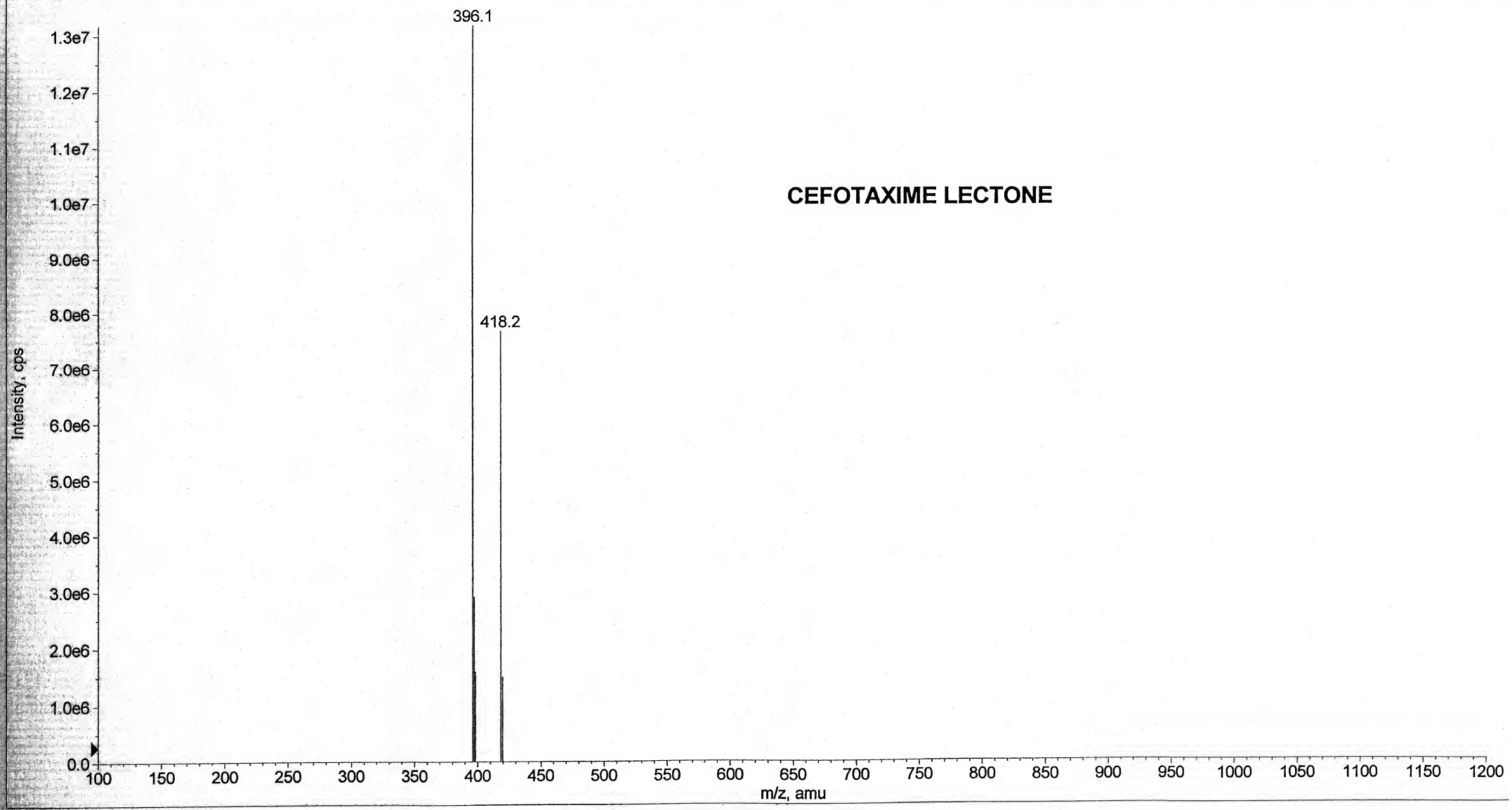
DEPT OF CEFTIOFUR LACTONE.



INSTRUMENT : PERKIN ELMER 1600 Series FTIR

Sample ID : Cefotaxime Lactone





Sample Name: CEFOTAXIME LECTONE

OBJECTIVE: - ISOLATION OF IMPURITY AT RRT ~ 0.37

This impurity at RRT ~ 0.37 was isolated from Ceftiofur sample using preparative HPLC.

Preparative isolation

The chromatographic conditions used was as follows:

Column

- Type: X-Terra C_{18}
- Dimensions: 300 mm x 19 mm
- Particle size: 7 μm

Detector setting

- Wavelength: 235 nm
- Flow rate: 15 ml/min

Mobile Phase

- Mobile A: Water
- Mobile B: Acetonitrile

Sample preparation:

~80 mg material dissolved in 8 ml of pH7.0 buffer, filtered and loaded on preparative column

Loading amount: 80 mg sample/injection

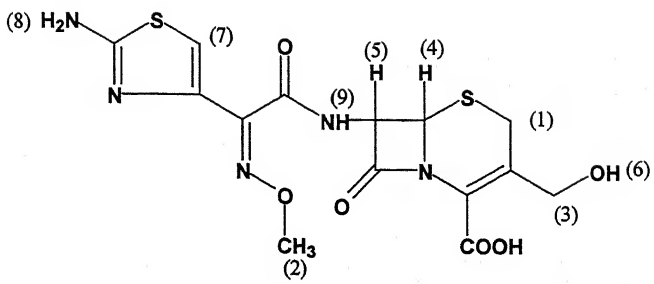
Chromatographic condition

S.No.	Time	Event	Value	Flow ml/min
1.	0.01	B.conc	0.00	15.0
2.	5.00	B.conc	0.00	15.0
3.	10.00	B.conc	15.00	15.0
4.	12.00	B.conc	50.00	15.0
5.	15.00	B.conc	100.00	15.0
6.	18.00	B.conc	100.00	15.0
7.	20.00	B.conc	0.00	15.0
8.	25.00	B.conc	0.00	15.0

Fractions collected were monitored using the analytical method. Fractions having the impurity sample in (~97%) purity were concentrated using rotary evaporator at bath temperature 35°C under vacuum to distill out acetonitrile. The combined fractions were then lyophilized to furnish ≥97 % (By HPLC area normalization method) pure material, as white solid.

The solid obtained was taken for further evaluation of the purity and also for various spectroscopic studies.

SPECTRAL ANALYSIS



S.No.	Chemical Shift (ppm)	Multiplicity	Relative number of protons	Proton Assignment
1.	9.5	d	1	9
2.	7.23	s	2	8
3.	6.73	s	1	7
4.	6.11	S	1	6
5.	5.57-5.51	dd	1	5
6.	4.93	d	1	4
7.	4.17-4.11	d	1	3 (1H)
8.	3.83	s	4	2+3(1H)
9.	3.29-3.20	q	2	1

IR in KBr

The IR spectrum was recorded on FTIR-8201PC Shimadzu instrument using KBr pallet.

S.No.	Frequency Cm^{-1}	Assignment
1.	3316.0	-OH
2.	3202.6	-NH
3.	2938.7	$-S-CH_2$
4.	1760.1	-C=O Lactam
5.	1664.3	-CONH
6.	1533.2	-CON-
7.	1036.0	-CO

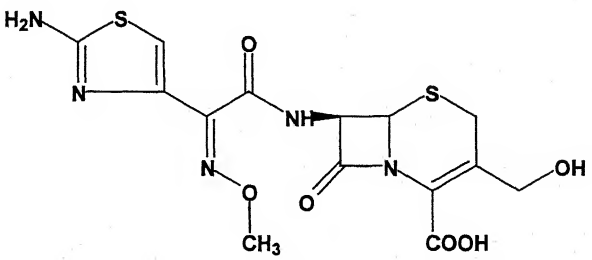
MASS SPECTRA

The compound exhibited a quasi-molecular ion peak at 414 implying a molecular weight of 413. For mass spectrum the sample was dissolved in methanol and spectrum was taken using Atmospheric Pressure Ionization technique having turbolon ionization source. The declustering potential was set at 6 v; focusing potential at 60v, and ionization volt energy (IVE) 4000 at 350°C

Molecular weight	Structure
414.1	$(M+H)^+$ Molecular ion
436.1	$(M+Na)^+$

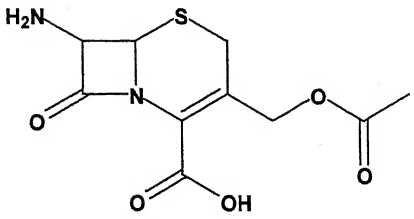
CONCLUSION

The unidentified impurity at RRT 0.37 was isolated from impurity-enriched synthetic sample using preparative HPLC. The structure of this impurity was assigned on the basis of NMR, IR and mass spectra. It was identified as Desacetyl cefotaxime,



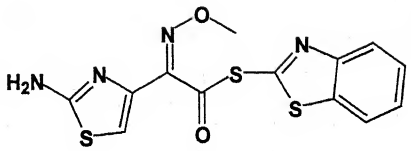
(6R)-6-[(2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza-3-methoxyprop-2-enoylamino]-3-(hydroxymethyl)-5-oxo-2H,6H,6aH-azetidino[2,1-b]1,3-thiazine-4-carboxylic acid. (Desacetyl Cefotaxime)

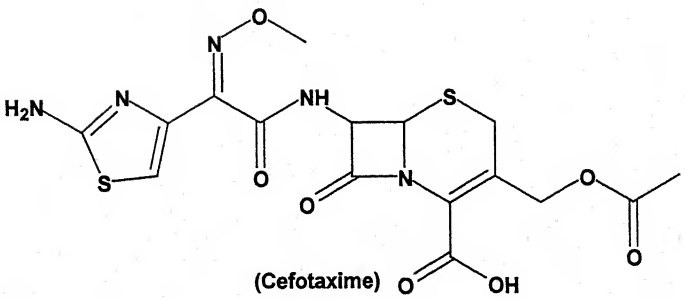
PROPOSED FORMATION PATHWAY



7-ACA
(Starting Material)

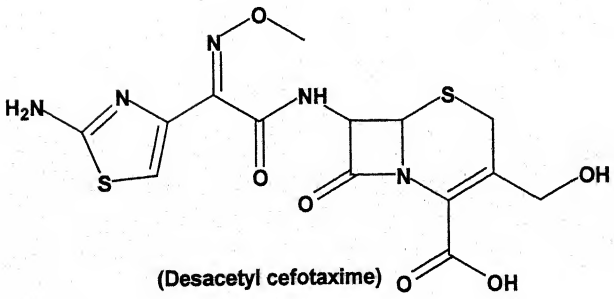
MAEM





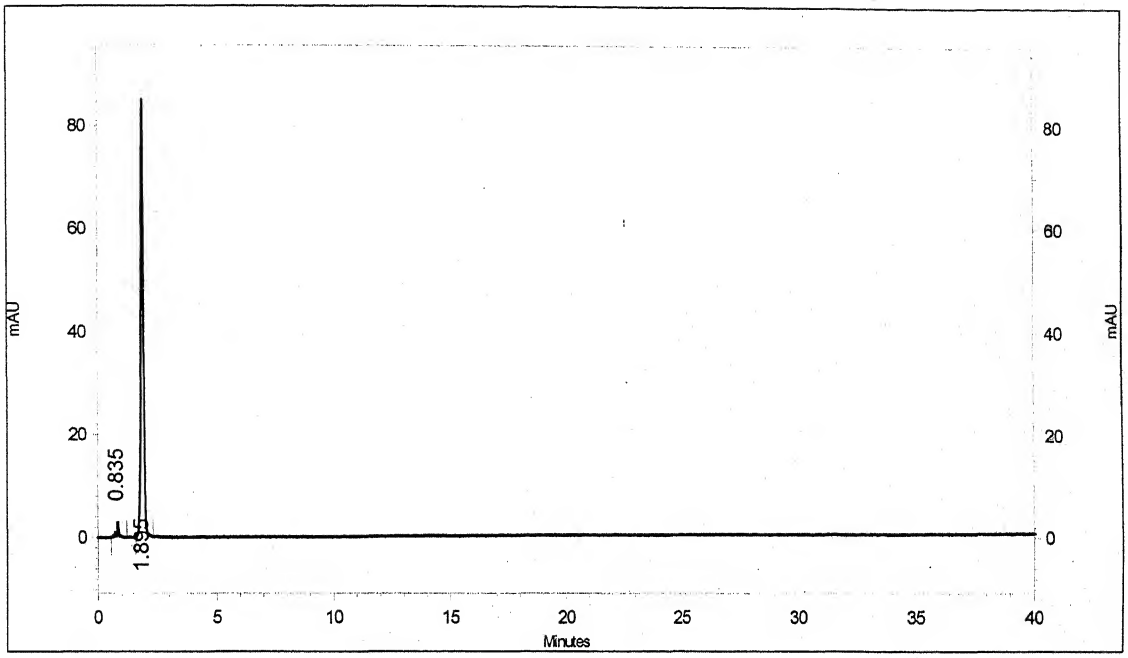
(Cefotaxime)

Base



(Desacetyl cefotaxime)

Method Name: E:\HPLC-24\Method\Ceftiofur\Ceftiofur 2.met
File Name: E:\HPLC-24\Data\Jan\Ceftiofur\24-013003.2
Aquired Time: 1/30/2003 10:47:17 AM
Sample ID: Desacetyl Cefotaxime



Detector 1-235nm
Results
(Reprocessed)

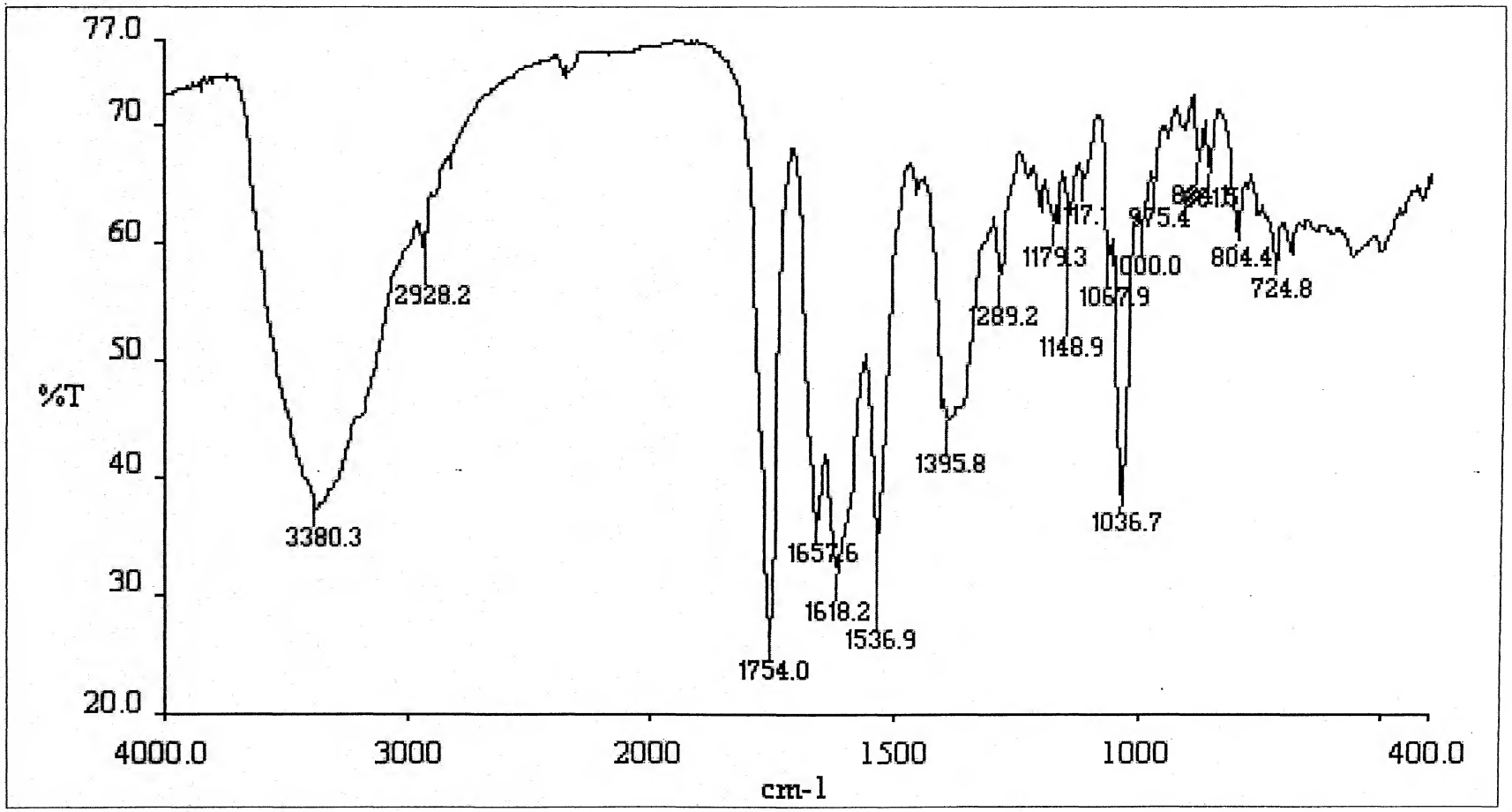
Pk #	Retention Time	Area	Area Percent	Name
1	0.835	18970	2.81	
2	1.895	657240	97.19	**Desacetyl Cefotaxime**

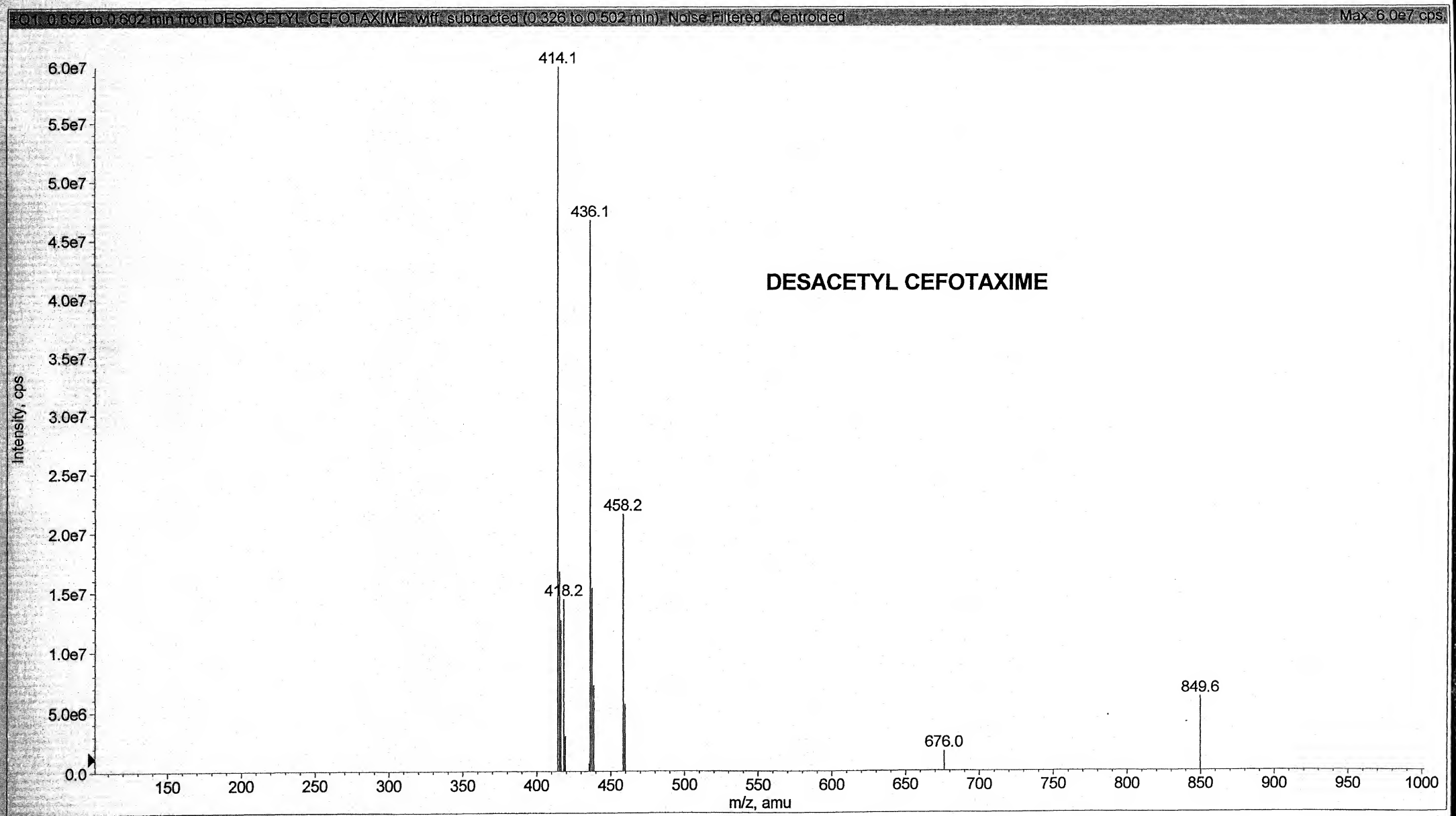
Totals		676210	100.00	

Desacetyl Cefotaxime in DMSO-d6

INSTRUMENT : PERKIN ELMER 1600 Series FTIR

Sample ID : Desacetyl Cefotaxime





Sample Name: DESACETYL CEFOTAXIME

OBJECTIVE: - ISOLATION OF IMPURITY AT RRT ~ 0.56

This impurity at RRT ~ 0.56 was isolated from Ceftiofur sample using preparative HPLC.

Preparative isolation

The chromatographic conditions used was as follows:

Column

- Type: X-Terra C_{18}
- Dimensions: 300 mm x 19 mm
- Particle size: 7 µm

Detector setting

- Wavelength: 235 nm
- Flow rate: 15 ml/min

Mobile Phase:

- Mobile A: Water
- Mobile B: Acetonitrile

Sample preparation

~80 mg material dissolved in 4 ml of pH7.0 buffer.

Loading amount: 80 mg sample/injection

Chromatographic condition

S.No.	Time	Event	Value	Flow ml/min
1.	0.01	B.conc.	0.00	15.0
2.	5.00	B.conc.	0.00	15.0
3.	10.00	B.conc.	15.00	15.0
4.	20.00	B.conc.	30.00	15.0
5.	25.00	B.conc.	50.00	15.0
6.	30.00	B.conc.	70.00	15.0
7.	35.00	B.conc.	0.00	15.0
8.	40.00	B.conc.	0.00	15.0

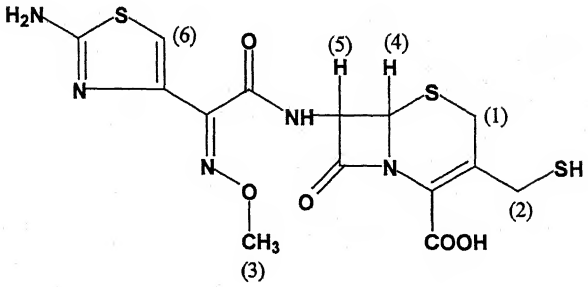
Fractions collected were monitored using the analytical method. Fractions having the impurity sample in (~98.0%) purity were concentrated using rotary evaporator at bath temperature 35°C under vacuum to distill out acetonitrile. The combined fractions were then lyophilized to furnish ≥98.0 % (By HPLC area normalization method) pure material, as white solid.

The solid obtained was taken for further evaluation of the purity and also for various spectroscopic studies.

SPECTRAL ANALYSIS

NMR SPECTRA

The NMR spectra of impurity at RRT ~ 0.56 was recorded on a 200 MHz Bruker DRX-200 instrument using CD_3OD solvent.



S.No.	Chemical Shift (ppm)	Multiplicity	Relative number of protons	Proton Assignment
1.	6.73	s	1	6
2.	5.64	d	1	5
3.	4.97	d	1	4
4.	3.86	s	3	3
5.	3.69-3.59	q	3	1+2 (1H)
6.	3.21	d	1	2 (1H)

IR in KBr

The IR spectrum was recorded on FTIR-8201PC Shimadzu instrument using KBr pallet.

S.No.	Frequency Cm^{-1}	Assignment
1.	3317.3	-OH
2.	3200	-NH
3.	2936.2	$-S-CH_2$
4.	1759.2	-C=O Lactam
5.	1665.7	-CONH
6.	1531.4	-CON-
7.	1037.7	-CO

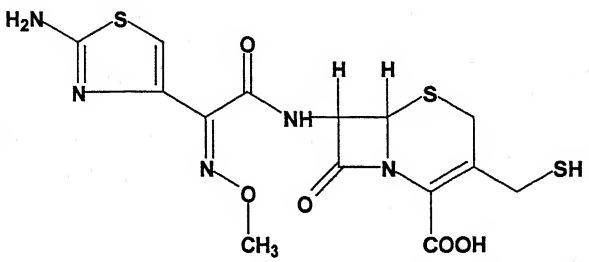
MASS SPECTRA

The compound exhibited a quasi-molecular ion peak at 430 implying a molecular weight of 429. The mass spectrum was recorded on PE SCIEX API-3000 triple quadrupole mass spectrometer. For mass spectrum the sample was dissolved in methanol and spectrum was taken using Atmospheric Pressure Ionization technique having turbolon ionization source. The declustering potential was set at 6 v; focusing potential at 60v, and ionization volt energy (IVE) 4000 at 350°C

S.No.	Molecular weight	Structure
1.	430.2	$(M+H)^+$ Molecular ion
2.	452.1	$(M+Na)^+$

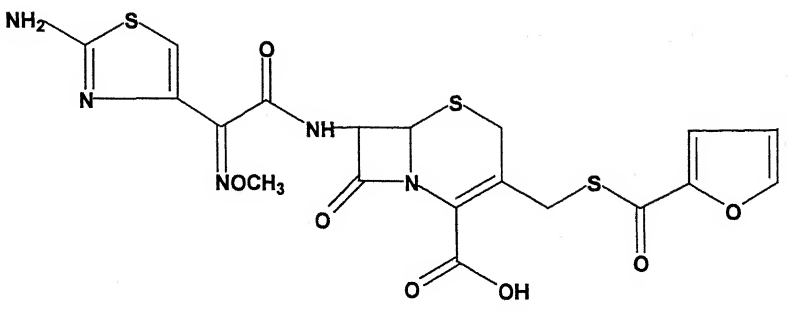
CONCLUSION

The unidentified impurity at RRT 0.56 was isolated from impurity-enriched synthetic sample using preparative HPLC. The structure of this impurity was assigned on the basis of NMR, IR and mass spectra. It was identified as Ceftiofur thiol,



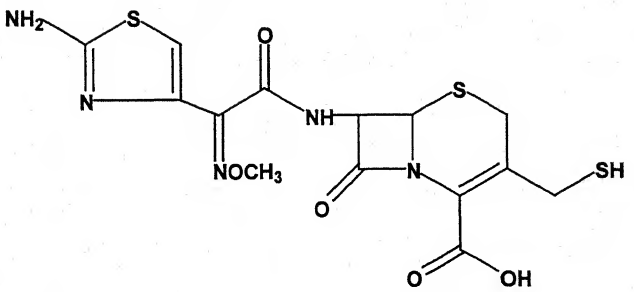
6-[(2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza-3-methoxyprop-2-enoylamino]-5-oxo-3-(sulfanylmethyl)-2H,6H,6aH-azetidino [2,1-b]1,3-thiazine-4-carboxylic acid. (Ceftiofur thiol)

PROPOSED FORMATION PATHWAY OF IMPURITY



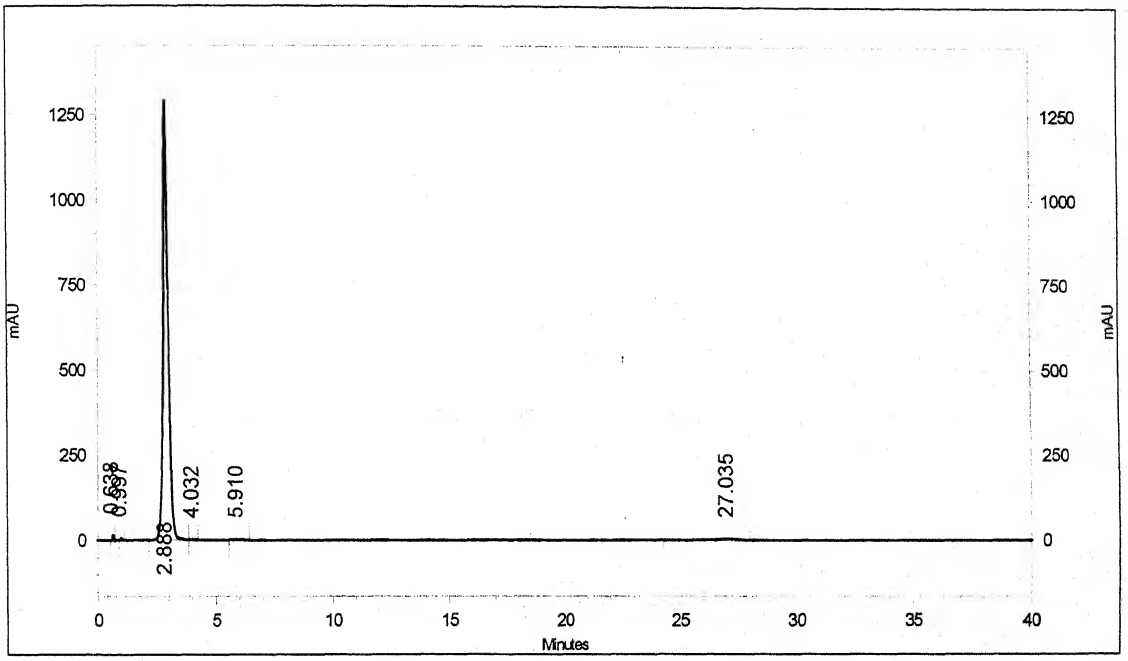
Ceftiofur

Base



Ceftiofur Thiol

Method Name: E:\HPLC-24\Method\Ceftiofur\Ceftiofur 2.met
File Name: E:\HPLC-24\Data\Jan\Ceftiofur\24-011003.5
Aquired Time: 1/10/2003 11:59:15 AM
Sample ID: Ceftiofur Thiol



Detector 1-235nm
Results
(Reprocessed)

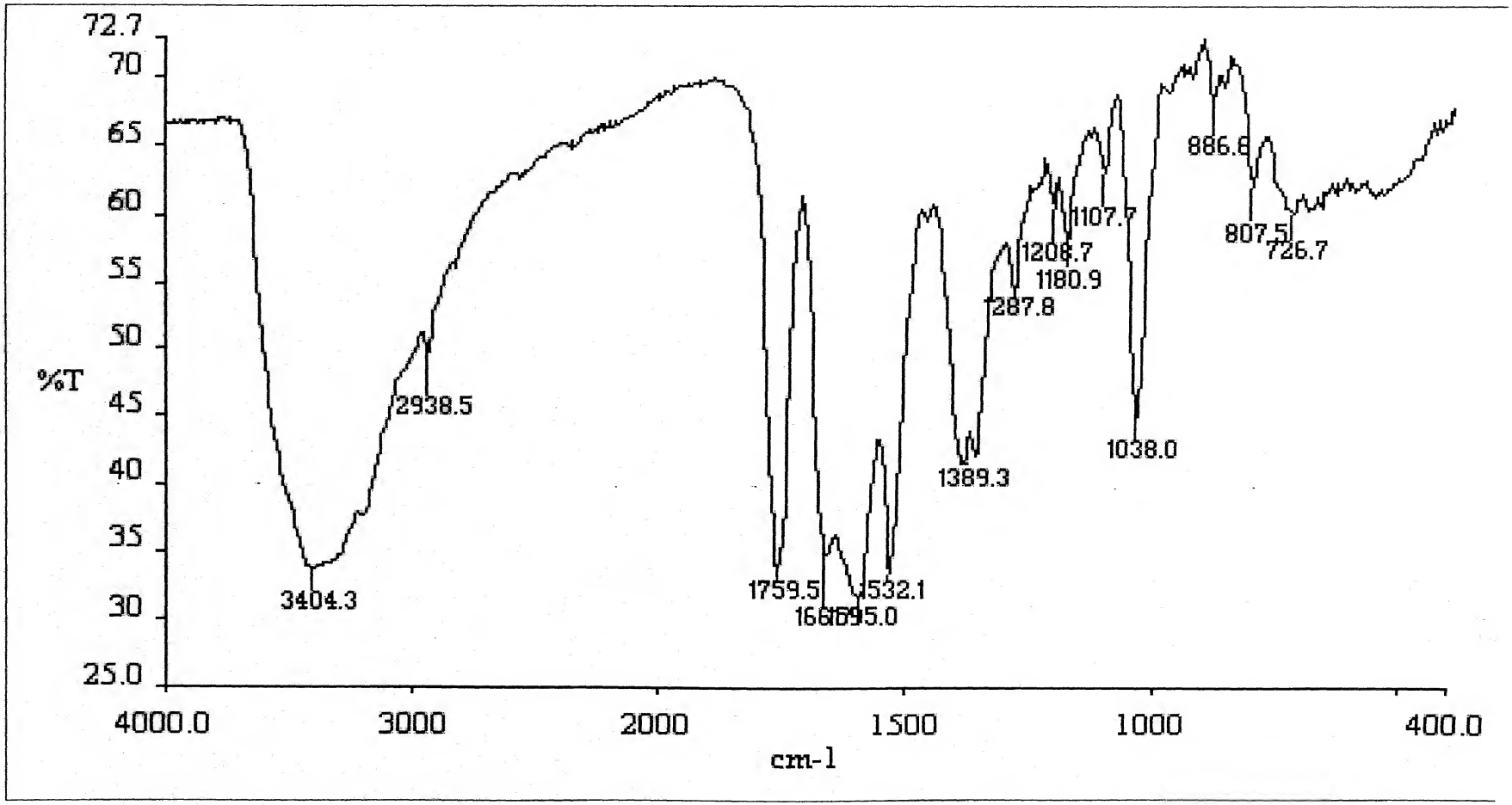
Pk #	Retention Time	Area	Area Percent	Name
1	0.638	65060	0.35	
2	0.997	20512	0.11	
3	2.888	18418065	98.06	**Ceftiofur Thiol**
4	4.032	6836	0.04	
5	5.910	22708	0.12	
6	27.035	249845	1.33	

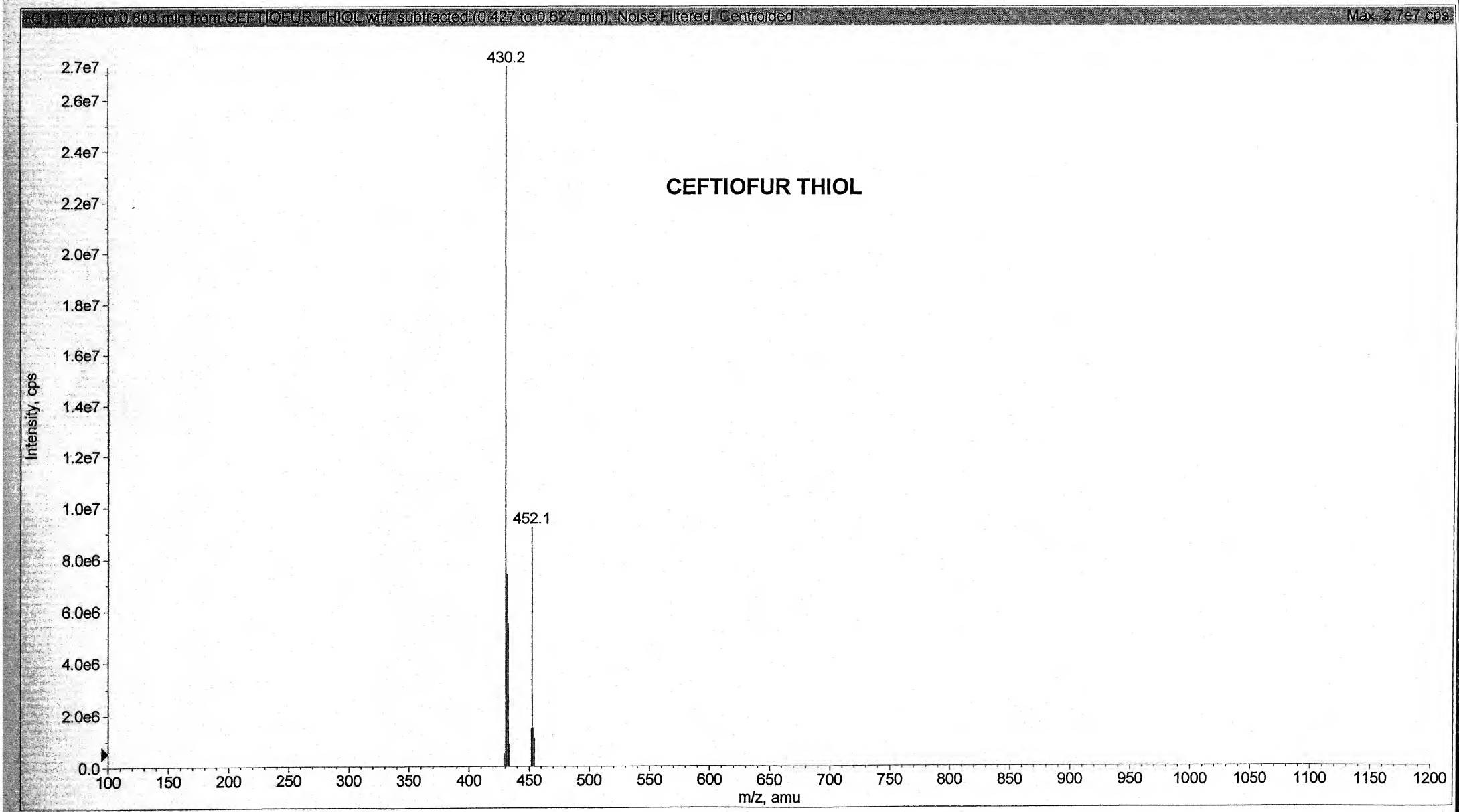
Totals		18783026	100.00	

Ceftiofur Thiol in MeOD
6.738
5.644
5.621
4.979
4.955
4.852
4.757
4.655
3.867
3.659
3.593
3.489
3.460
3.216
3.208
3.200
3.193
3.185
3.087
3.021
2.518
1.091
1.000
1.315
0.828
1.461
1.450
3.690
ppm

INSTRUMENT : PERKIN ELMER 1600 Series FTIR

Sample ID : Ceftiofur Thiol





98

Sample Name: CEFTIOFUR THIOL

OBJECTIVE: - ISOLATION OF IMPURITY AT RRT ~ 1.58

This impurity at RRT ~1.58 was isolated from Ceftiofur sample using preparative HPLC.

Preparative isolation

The chromatographic condition used was as follows:

Column

- Type: X-Terra C_{18}
- Dimensions: 300 mm x 19 mm
- Particle size: 7 μm

Detector setting

- Wavelength: 235 nm
- Flow rate: 15 ml/min

Mobile Phase:

- Mobile A: Water
- Mobile B: Acetonitrile

Sample preparation

~100 mg material dissolved in 4 ml of water and one drop of dilute ammonia (10%) and loaded on preparative column

Loading amount: 100 mg sample/injection

Chromatographic condition

S.No.	Time	Event	Value	Flow ml/min
1.	0.01	B.conc	0.00	15.0
2.	5.00	B.conc	0.00	15.0
3.	10.00	B.conc	30.00	15.0
4.	15.00	B.conc	50.00	15.0
5.	20.00	B.conc	70.00	15.0
6.	30.00	B.conc	70.00	15.0
7.	35.00	B.conc	40.00	15.0
8.	40.00	B.conc	0.00	15.0

Fractions collected were monitored using the analytical method. Fractions having the impurity sample in (~93%) purity were concentrated using rotary evaporator at bath temperature 35°C under vacuum to distill out acetonitrile. The combined fractions were then lyophilized to furnish ≥93.0 % (By HPLC area normalization method) pure material, as white solid.

The solid obtained was taken for further evaluation of the purity and also for various spectroscopic studies.

SPECTRAL ANALYSIS

NMR SPECTRA

The NMR spectra of impurity at RRT ~1.58 was recorded on a 200 MHz Bruker DRX-200 instrument using $CDCl_3$ solvent.

1H NMR

7.71 (s 2H); 7.29 (d 2H); 6.65 (d, 2H) ppm/δ

IR in KBr

The IR spectrum was recorded on FTIR-8201PC Shimadzu instrument using KBr pallet.

954.9; 883.8; 822.8; 765.0 cm^{-1}

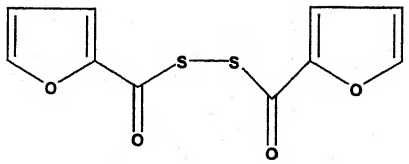
MASS SPECTRA

The compound exhibited a quasi-molecular ion peak at 255 implying a molecular weight of 254. For mass spectrum the sample was dissolved in methanol and spectrum was taken using Atmospheric Pressure Ionization technique having turbolon ionization source. The declustering potential was set at 6 v; focusing potential at 60v, and ionization volt energy (IVE) 4000 at 350°C.

Molecular weight	Structure
255.3	$(M+H)^+$ Molecular ion

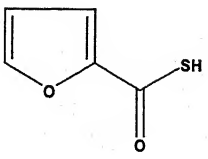
CONCLUSION

Spectroscopic (IR, NMR, MS) analysis of the compound identified as 2-furyl (2-furylcarbonyl) disulfanyl ketone (Disulfide of thiofuric acid),

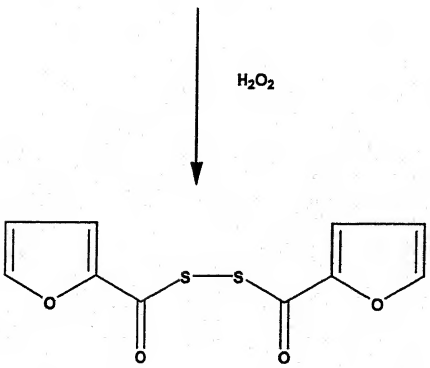


2-furyl (2-furylcarbonyl) disulfanyl ketone

PROPOSED FORMATION PATHWAY OF IMPURITY

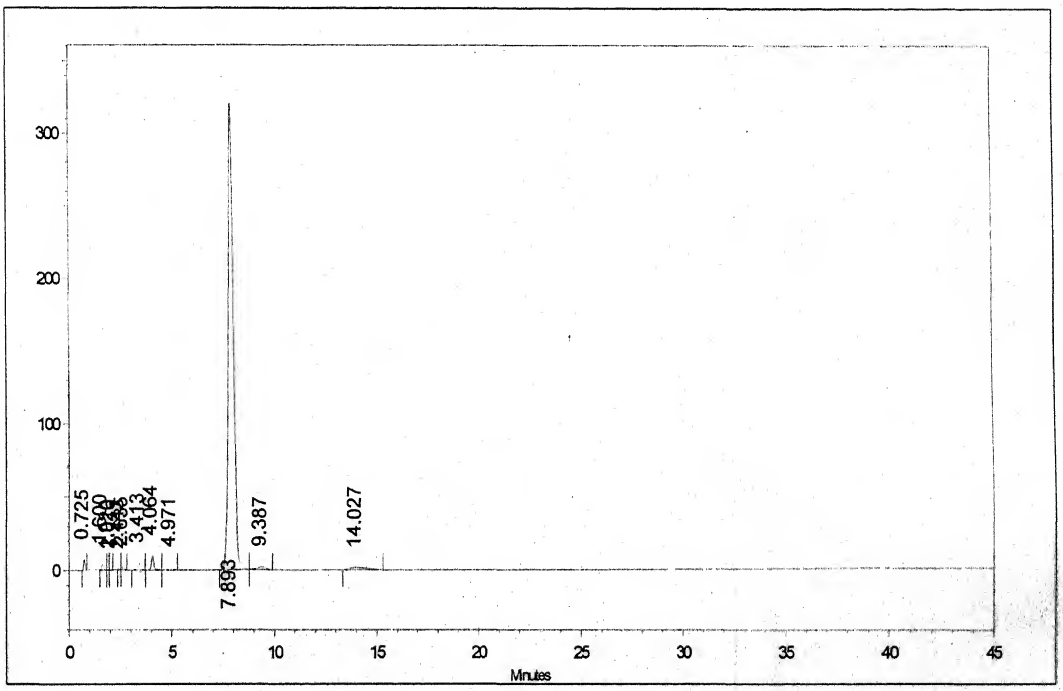


Thiofuroic Acid



TFA Disulfide

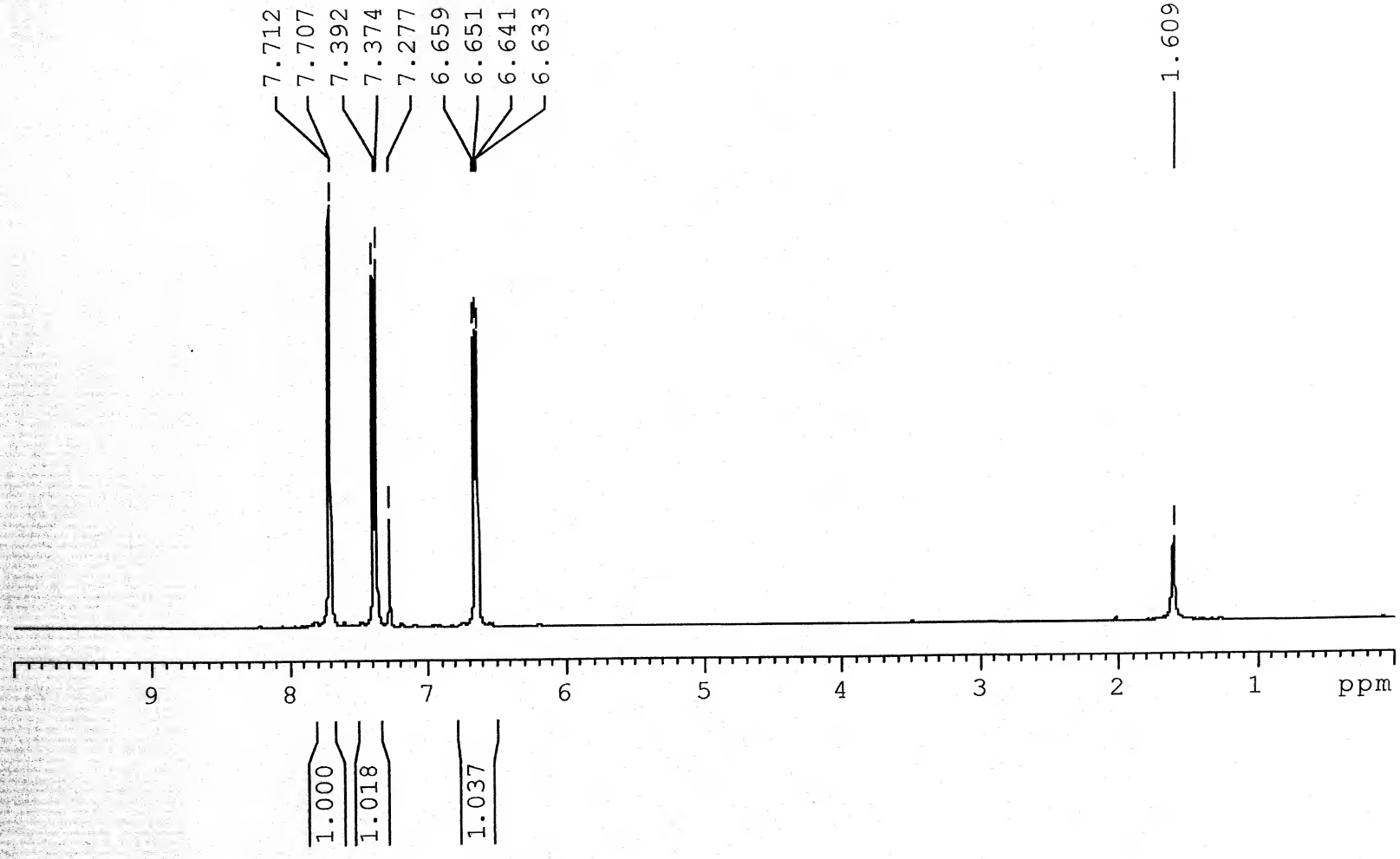
Method Name: D:\CLASSvp\PDA-LC33\Method\Ceftiofur\035_THAB.met
File Name: D:\CLASSvp\PDA-LC33\Data\Feb-04\Ceftiofur\022804.24
Aquired Time: 2/29/2004 8:12:36 AM
Sample ID: Disulfide of Thiofuric Acid



1: 235 nm, 8 nm

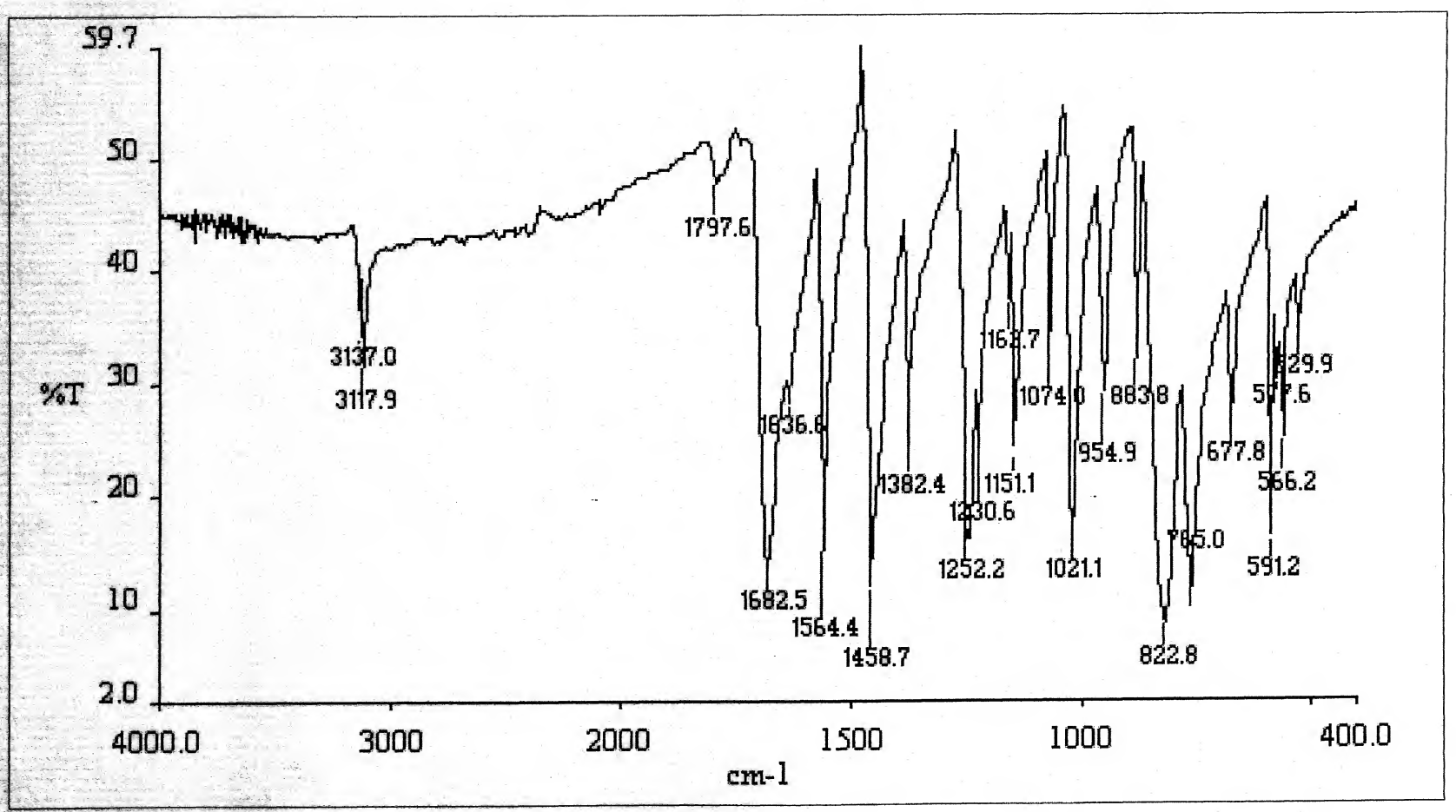
Pk #	Retention Time	Area	Area Percent	Name
1	0.725	35325	0.59	
2	1.600	26023	0.43	
3	1.920	1027	0.02	
4	2.016	3281	0.05	
5	2.443	2806	0.05	
6	2.635	15017	0.25	
7	3.413	49945	0.83	
8	4.064	83502	1.39	
9	4.971	23825	0.40	
10	7.893	5608274	93.58	Disulfide of Thiofuric Acid
11	9.387	44430	0.74	
12	14.027	99617	1.66	
Totals		5993072	100.00	

TFA Disulfide in CDCL3



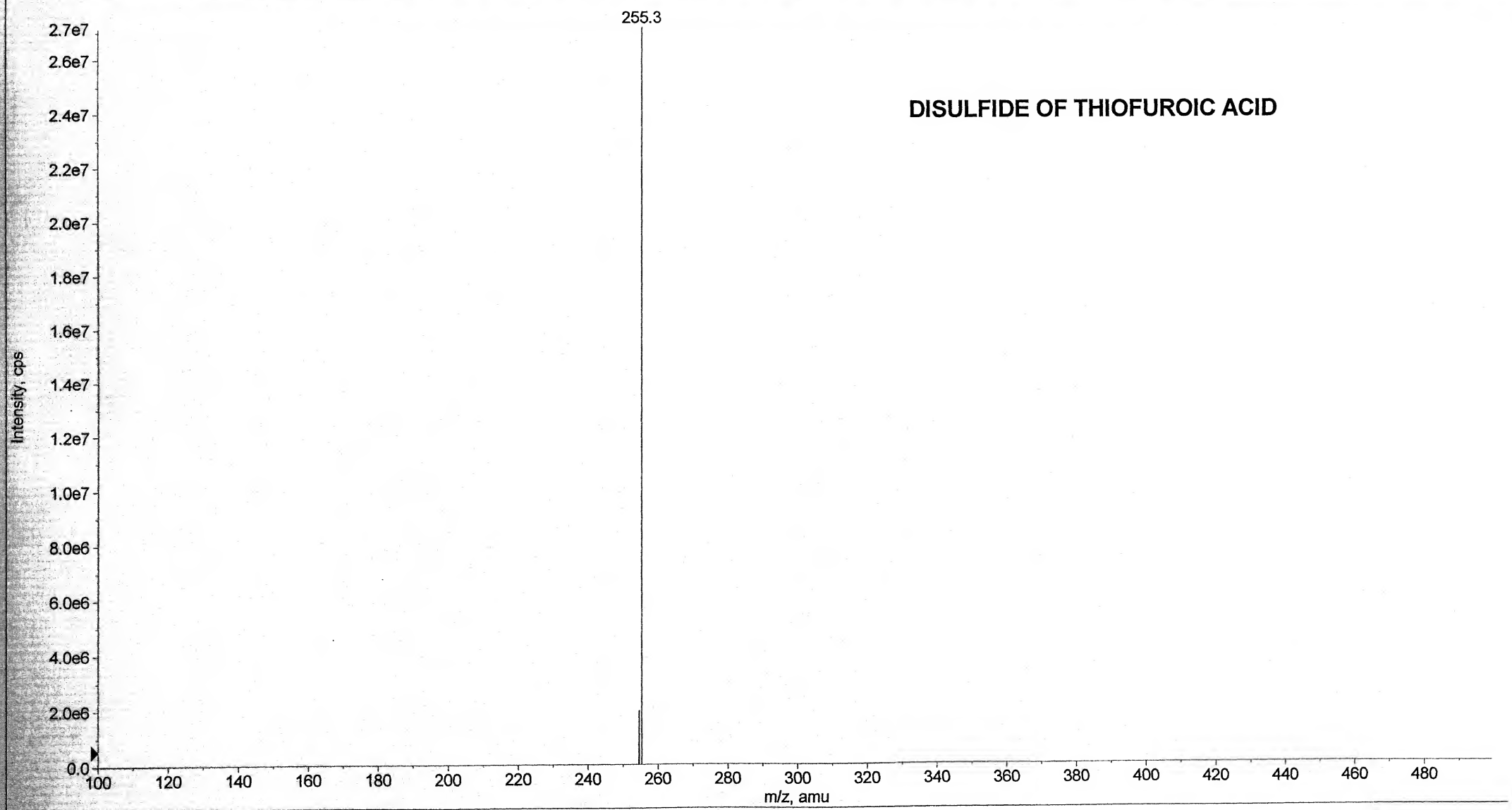
INSTRUMENT : PERKIN ELMER 1600 Series FTIR

Sample ID : TFA Disulfide



106

#Q1: 0.486 to 0.503 min from Sample 1 (DISULFIDE OF THIOFUROIC ACID) of DISULFIDE OF THIOFUROIC ACID.wiff, subtracted (0.251 to 0.452 mi... Max. 2.7e7 cps.



Sample Name: DISULFIDE OF THIOFUROIC ACID

OBJECTIVE: - ISOLATION OF IMPURITY AT RRT ~ 5.45

This impurity at RRT ~ 5.45 was isolated from Ceftiofur sample using preparative HPLC.

Preparative isolation

The chromatographic conditions used were as follows:

Column

- Type: X-Terra C_{18}
- Dimensions: 300 mm x 19 mm
- Particle size: 7 μm

Detector setting

- Wavelength: 235 nm
- Flow rate: 15 ml/min

Mobile Phase

- Mobile A: Water
- Mobile B: Acetonitrile

Sample preparation

To ~100 mg material dissolved in 4ml of water, add 1 drop of dil Ammonia and filter.

Loading amount: 100 mg sample/injection

Chromatographic condition

S.No.	Time	Event	Value	Flow ml/min
1.	0.01	B.conc	0.00	15.0
2.	5.00	B.conc	0.00	15.0
3.	10.00	B.conc	15.00	15.0
4.	20.00	B.conc	30.00	15.0
5.	25.00	B.conc	50.00	15.0
6.	30.00	B.conc	70.00	15.0
7.	35.00	B.conc	0.00	15.0
8.	40.00	B.conc	0.00	15.0

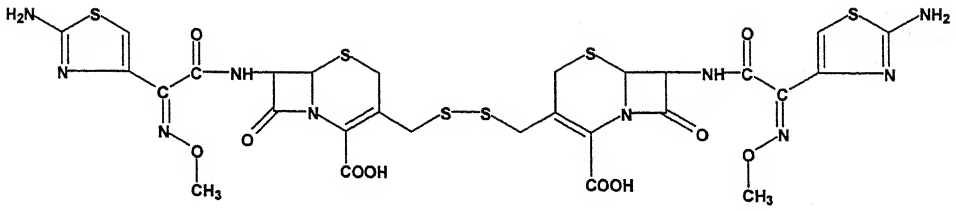
Fractions collected were monitored using the analytical method. Fractions having the impurity sample in (~99.0%) purity were concentrated using rotary evaporator at bath temperature 35°C under vacuum to distill out acetonitrile. The combined fractions were then lyophilized to furnish ≥99.00 % (By HPLC area normalization method) pure material, as white solid.

The solid obtained was taken for further evaluation of the purity and also for various spectroscopic studies.

SPECTRAL ANALYSIS

NMR SPECTRA

The NMR spectra of impurity at RRT ~ 5.45 was recorded on a 200 MHz Bruker DRX-200 instrument using DMSO-d6 and DMSO-d6+DCl solvent.



1H NMR

9.6 (d 2H); 7.2 (b, 4H); 6.73 (s, 2H); 5.67 (q, 2H); 5.09 (d, 2H) ppm/δ

The region between 3.0 to 4.5 is not clear due to interference of water therefore new spectrum was taken in mixture of DMSO-d6 and DCl. The obtained spectra clearly shows signal at 3.9 (s, 6H), and 3.4 – 3.7 (q, 4H) ppm/δ.

IR in KBr

The IR spectrum was recorded on FTIR-8201PC Shimadzu instrument using KBr pallet.

S.No.	Frequency Cm^{-1}	Assignment
1.	3315.4	-OH
2.	3203.5	-NH
3.	2939.3	$-S-CH_2$
4.	1764.7	-C=O Lactam
5.	1660.5	-CONH
6.	1531.4	-CON-
7.	1037.6	-CO

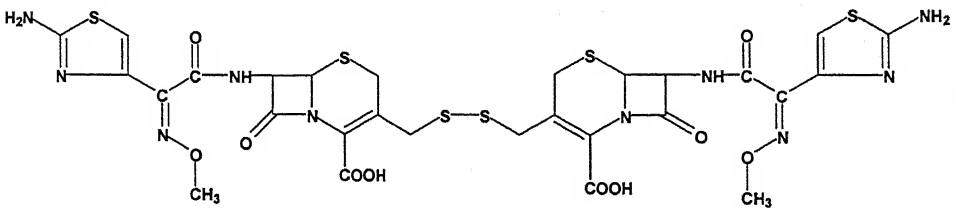
MASS SPECTRA

The compound exhibited a quasi-molecular ion peak at 857.3 implying a molecular weight of 856. For mass spectrum the sample was dissolved in methanol and spectrum was taken using Atmospheric Pressure Ionization technique having turbolon ionization source. The declustering potential was set at 6 v; focusing potential at 60v, and ionization volt energy (IVE) 4000 at 350°C.

Molecular weight	Structure
857.5	$(M+H)^+$ Molecular ion

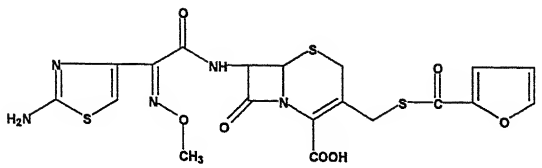
CONCLUSION

The unidentified impurity at RRT 5.45 was isolated from impurity-enriched synthetic sample using preparative HPLC. The structure of this impurity was assigned on the basis of NMR, IR and mass spectra. It was identified as Ceftiofur Disulfide,



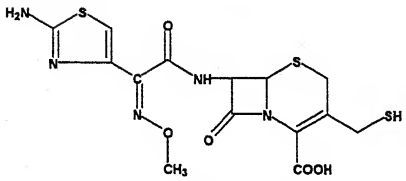
6-[(2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza-3-methoxyprop-2-enoylamino]-3-{[({6-[(2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza-3-methoxyprop-2-enoylamino]-4-carboxy-5-oxo(2H,6H,6aH-azetidino[2,1-b]1,3-thiazin-3-yl)}methyl)disulfanyl]methyl}-5-oxo-2H,6H,6aH-azetidino[2,1-b]1,3-thiazine-4-carboxylic acid

PROPOSED FORMATION PATHWAY OF IMPURITY



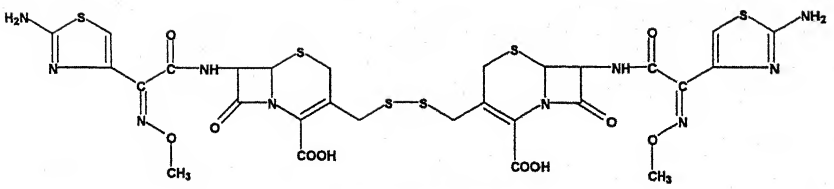
CEFTIOFUR

BASE



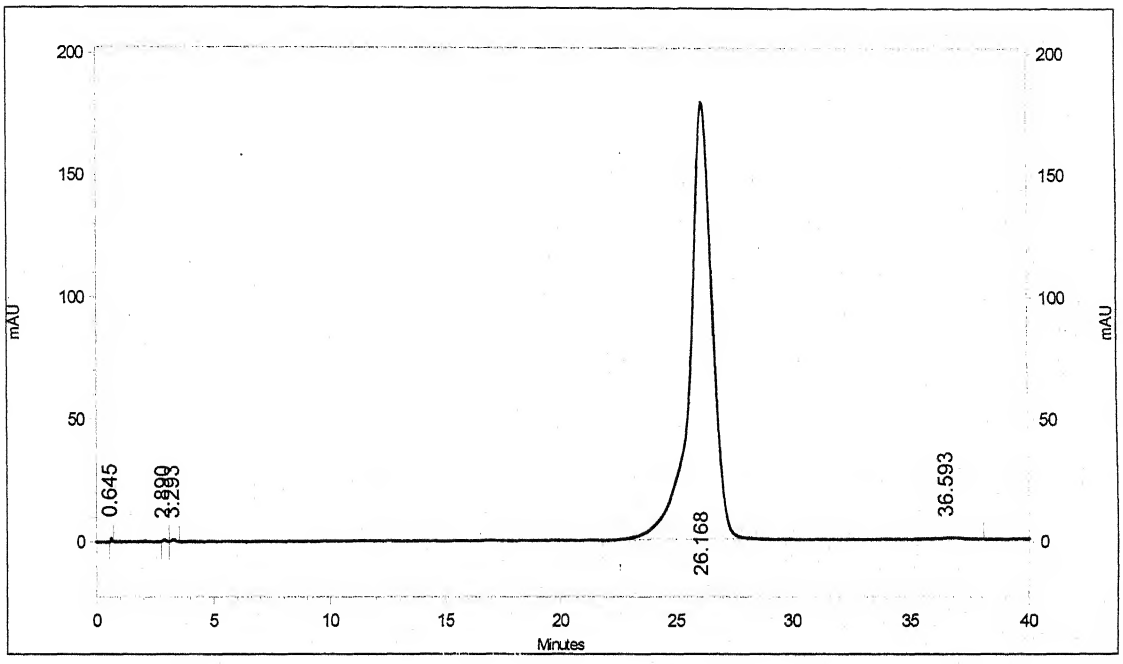
DESFUROYL CEFTIOFUR

[o]



DISULFIDE OF CEFTIOFUR

Method Name: E:\HPLC-24\Method\Ceftiofur\Ceftiofur 2.met
File Name: E:\HPLC-24\Data\Jan\Ceftiofur\24-011403.A2
Aquired Time: 1/14/2003 10:59:04 AM
Sample ID: Ceftiofur Disulfide



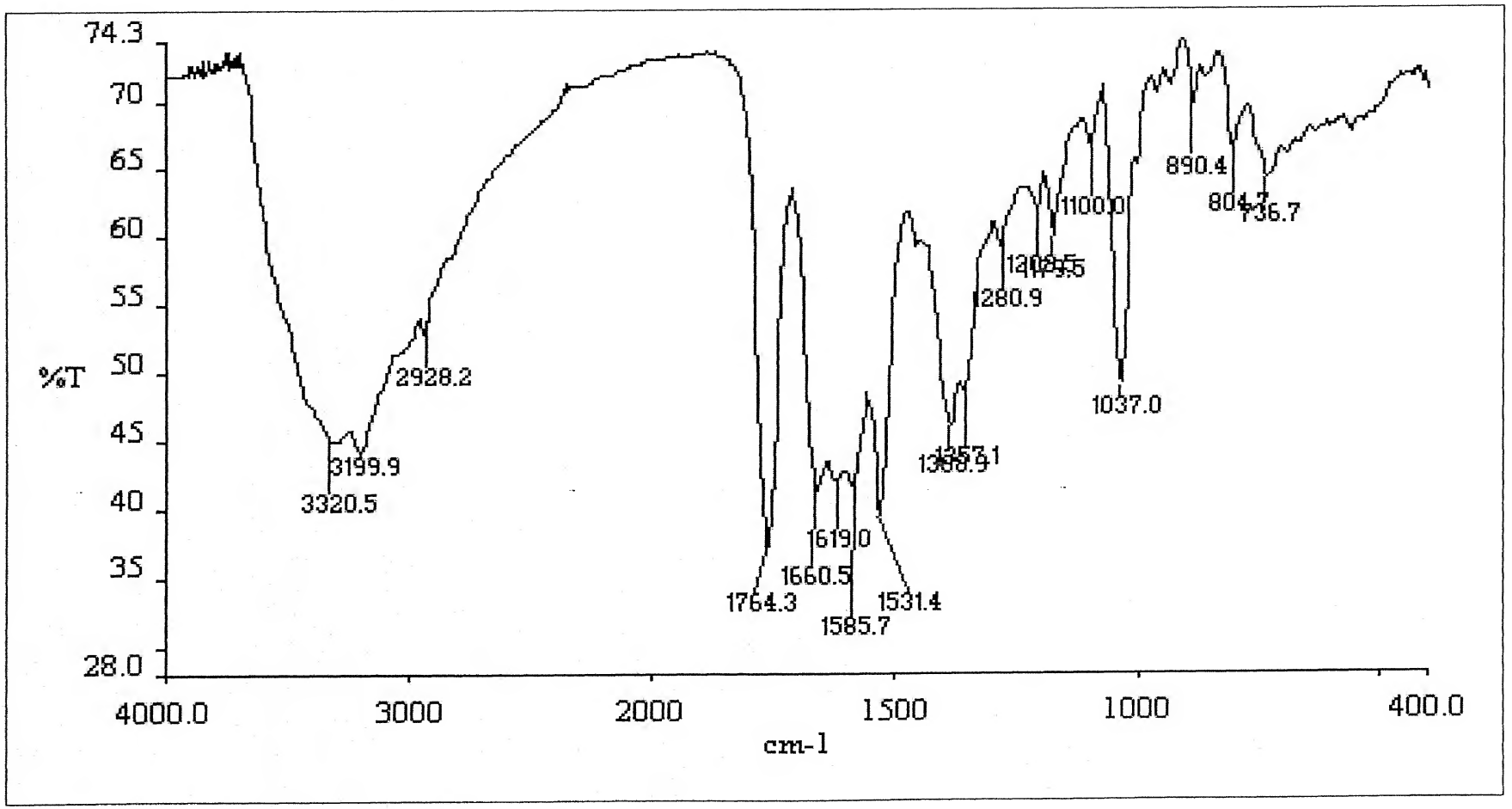
Detector 1-235nm Results (Reprocessed)

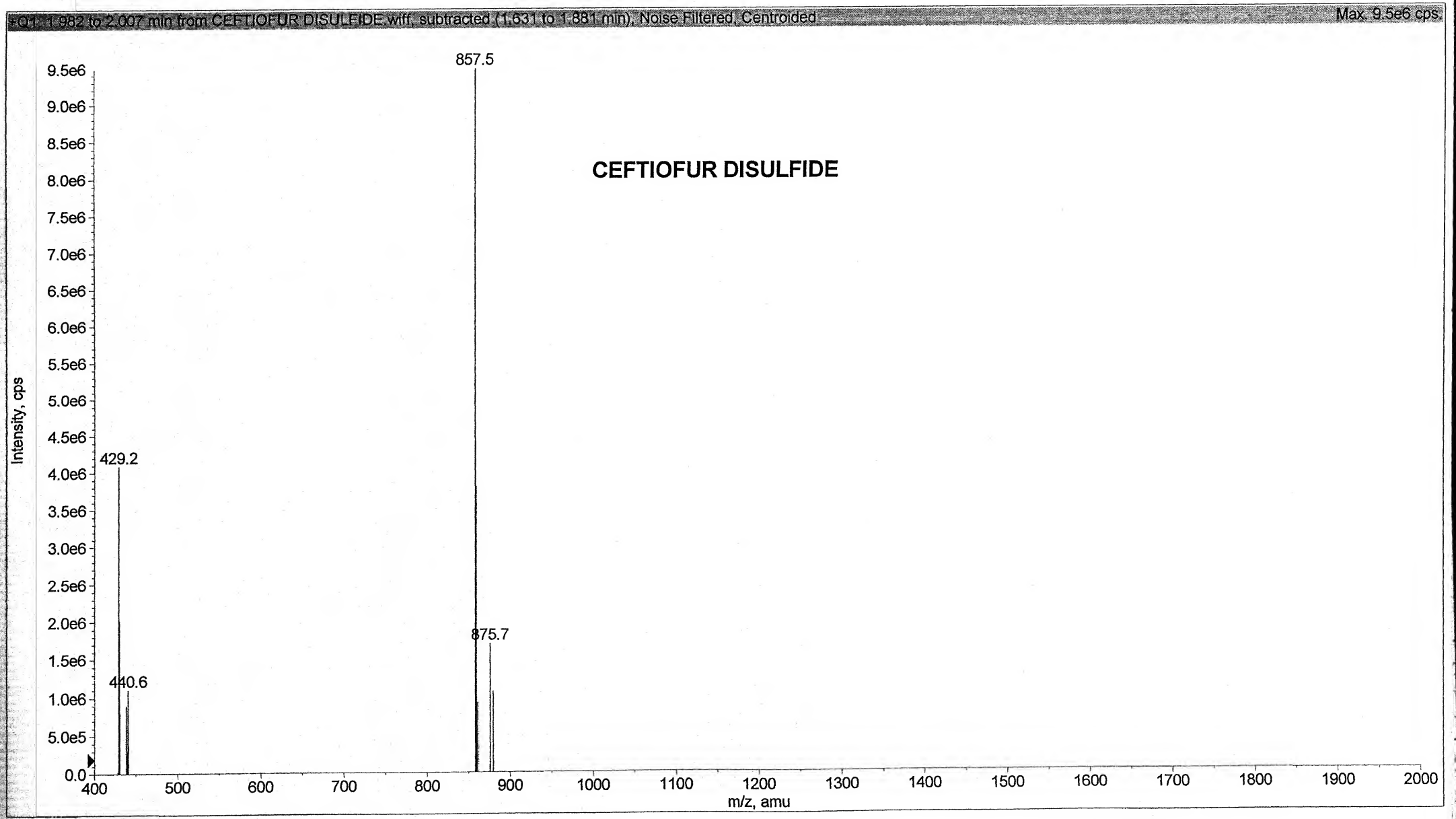
Pk #	Retention Time	Area	Area Percent	Name
1	0.645	5349	0.04	
2	2.890	6110	0.05	
3	3.293	8841	0.07	
4	26.168	12001726	99.53	**Ceftiofur Disulfide**
5	36.593	36043	0.30	
Totals		12058069	100.00	

Ceftiofur Disulfide in DMSO-d6
9.609
9.569
7.233
6.733
5.676
5.653
5.637
5.613
5.093
5.070
4.230
4.168
3.829
3.804
3.704
3.616
3.561
3.498
3.400
3.312
2.499
0.881
3.952
1.000
0.961
1.015
2.413
7.762
4.200
ppm

INSTRUMENT : PERKIN ELMER 1600 Series FTIR

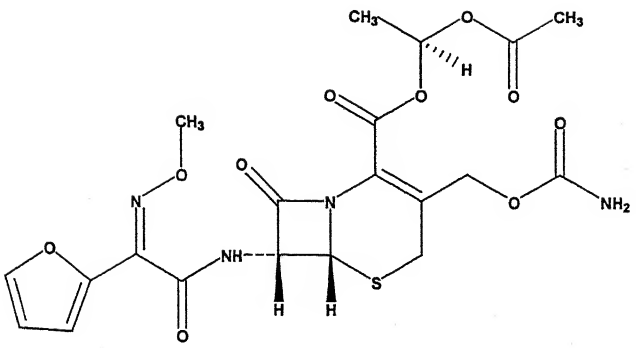
Sample ID : Ceftiofur Disulfide





Sample Name: CEFTIOFUR DISULFIDE

Product: **Cefuroxime Axetil**



Chemical name: - [6R-[6alpha, 7beta(Z)]]-3-[[(aminocarbonyl) oxy] methyl]-7-[[2-furanyl(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2carboxylicacid 1-(acetyloxy)ethyl ester.

Brand: Ceftin (Glaxo: Canada, USA)

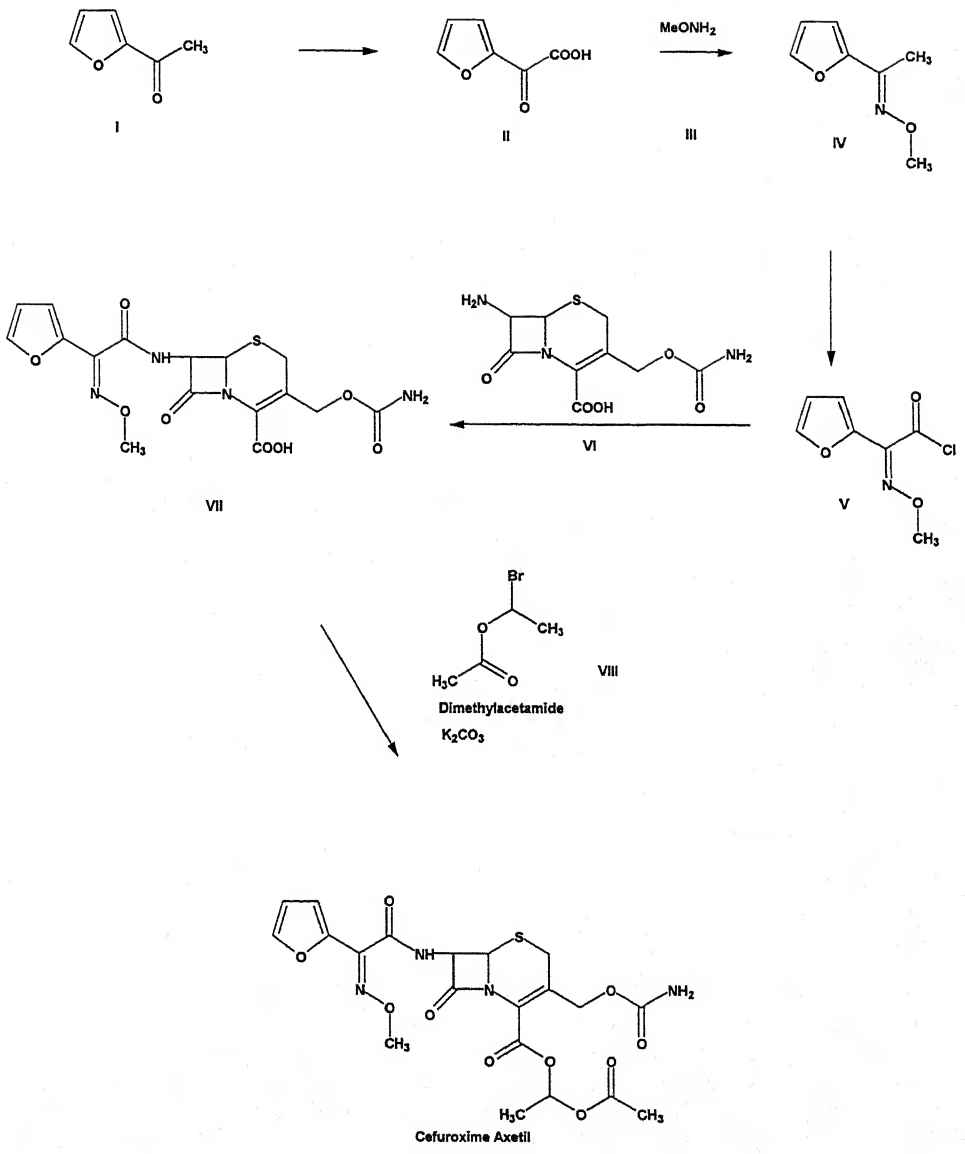
Molecular formula: $C_{20}H_{22}N_4O_{10}S$

Molecular Weight: 510.5

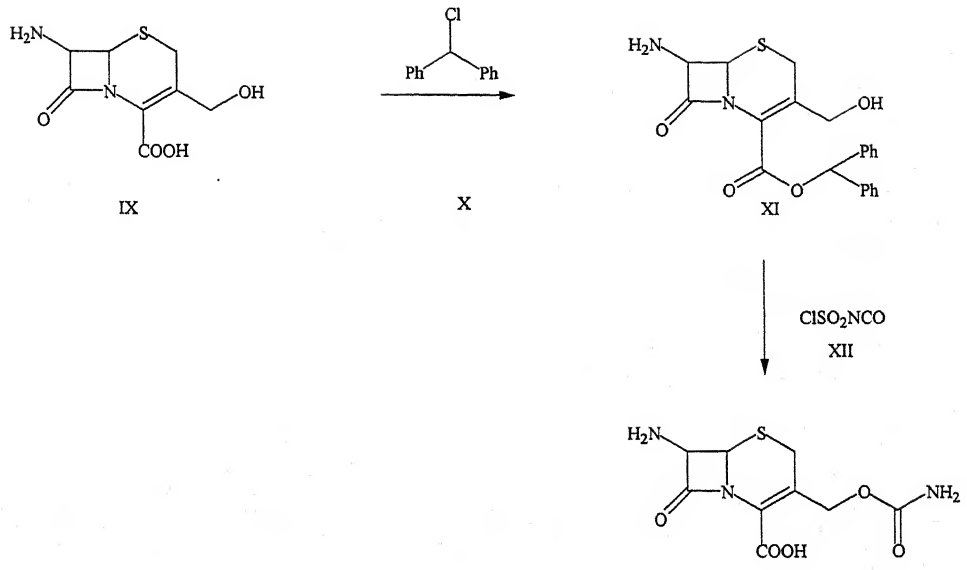
Uses: Antibacterial

Chemical Class: Second generation Beta-lactam antibiotics; Cephalosporin

SYNTHETIC SCHEME



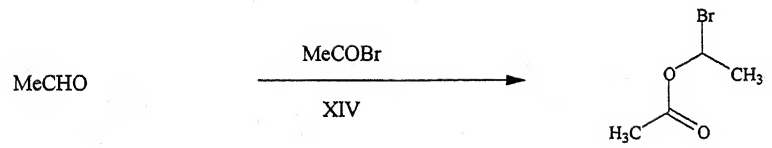
Preparation of VI



IX X XI

VI

Perparation of VIII



XII VIII

Intermediate used:

I. 2-acetylfuran

II. 2-furanyloxoacetic acid

III. O-methylhydroxylamine

IV. 2-furanyl(methoxyimino) acetic acid

V. 2-furanyl(methoxyimino) acetyl chloride

VI. 7-amino-3-[(carbamoyloxy)methyl]-3-cephem-4-carboxylic acid

VII. 3-[[(aminocarbonyl) oxy] methyl]-7-[[2-furanyl (methoxyimino) acetyl] amino]-8-oxo-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid.

VIII. Acetic acid 1-bromoethyl ester

IX. 7-amino-3-(hydroxymethyl)-3-cephem-4-carboxylic acid

X. chlorodiphenylmethane

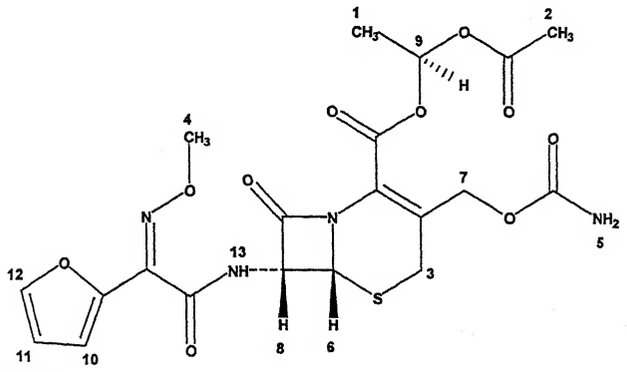
XI. 7-amino-3-(hydroxymethyl)-3-cephem-4-carboxylic acid diphenylmethyl ester.

XII. Chlorosulfonyl isocyanate

XIII. Acetaldehyde

XIV. Acetyl bromide

CHARACTERIZATION OF CEFUROXIME AXETIL



^{1}H NMR in $CDCl_3$

δ (ppm)	Relative protons	Assignment
7.48	1	13
7.32	1	12
7.11-6.98	1	10-11
6.88	1	9
6.46	1	8
5.96	1	7
5.11	2	6
4.77	3	5
4.05	3	4
3.56-3.46	2	3
2.08	3	2
1.53	3	1

IR in KBr

cm^{-1}	Functional group
3499.2	-OH stretch
3277.4	-N-H stretch
2938.9	S-CH_2
1779.4	ß-lactam C=O
1654.7	CONH

MASS SPECTRUM DATA

Mass	Assignment
528.9	$(M+NH_4)^+$

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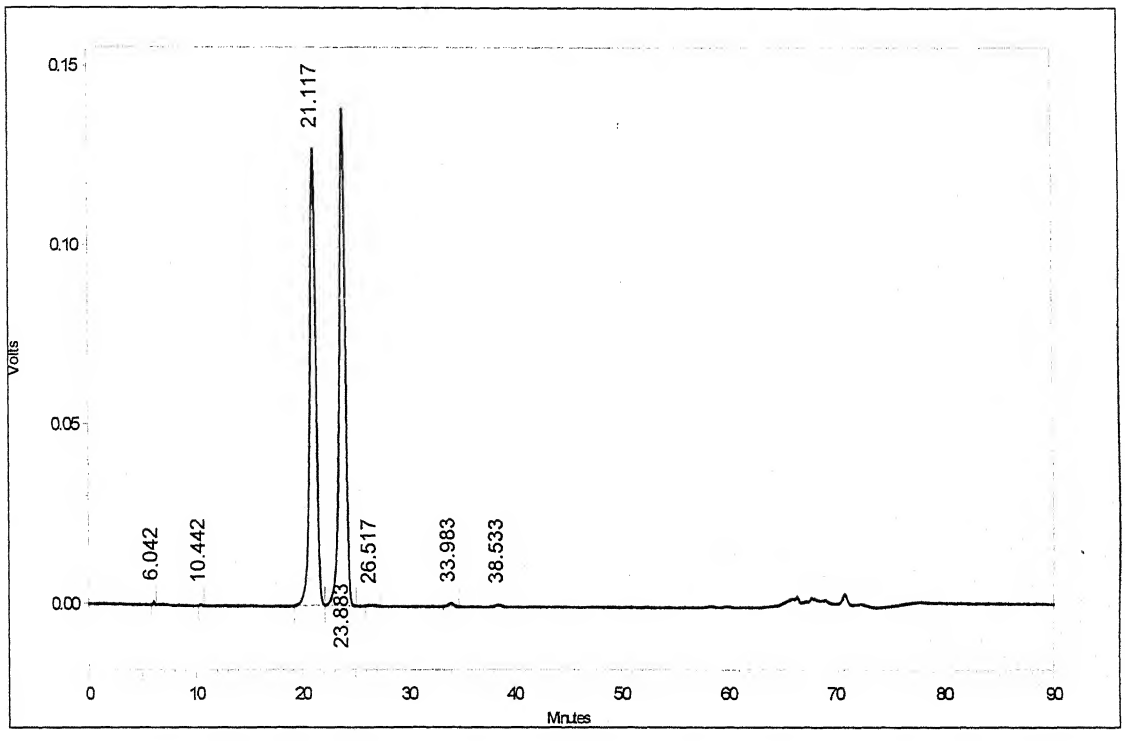
File Name: C:\HPLC-23\Data\Feb.04\Cef.Axetil\021304.00
Aquired Time: 2/13/2004 11:17:29 AM
Sample ID: Cefuroxime Axetil

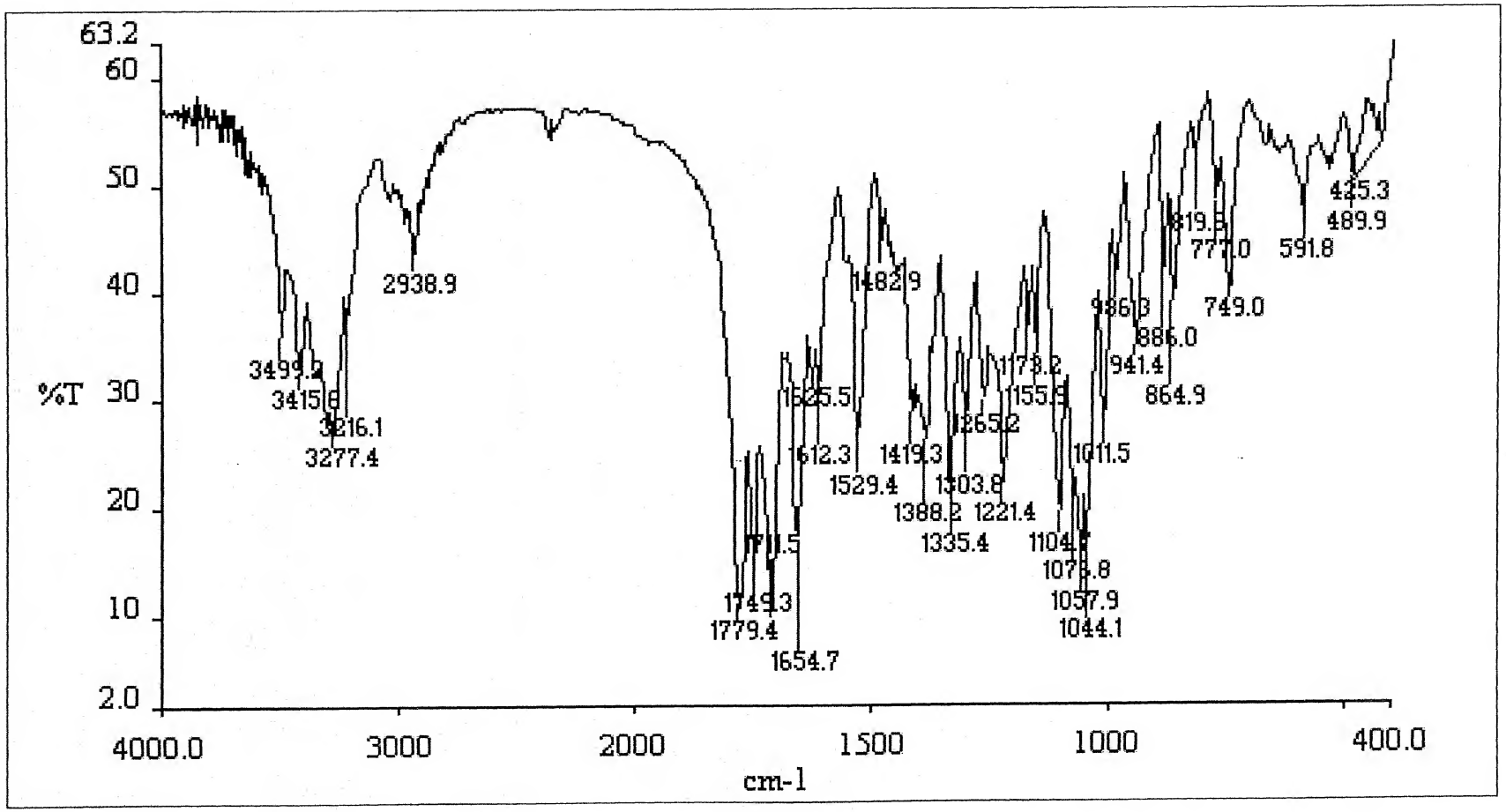


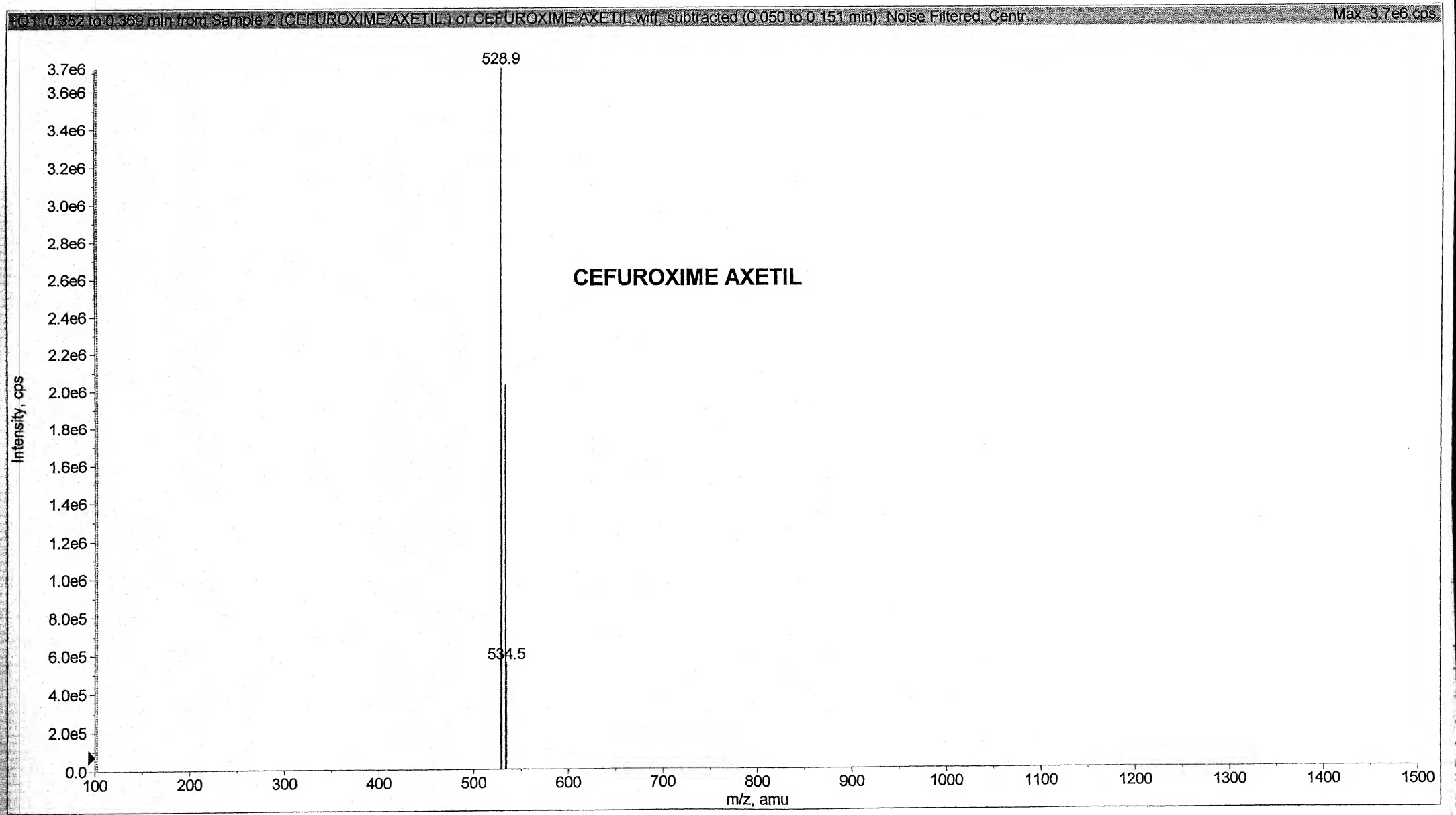
Detector A (278nm) Pk #	Retention Time	Area	Area Percent	Relative RT	Name
1	6.042	7348	0.07	0.28	
2	10.442	4401	0.04	0.49	
3	21.117	5092076	47.68	1.00	Isomer A
4	23.883	5512006	51.61	1.13	Isomer B
5	26.517	10302	0.10	1.26	
6	33.983	31710	0.30	1.61	
7	38.533	21292	0.20	1.82	
Totals		10679135	100.00		

Cefuroxime Axetil in CDCl3
7.486
7.481
7.324
7.280
7.238
7.113
7.086
6.983
6.956
6.882
6.862
6.841
6.463
6.455
6.446
6.437
5.969
5.947
5.925
5.119
5.089
5.053
5.030
5.020
4.848
4.816
4.779
4.748
4.057
3.562
3.477
3.461
2.080
2.076
1.616
1.542
1.530
1.515
1.502
ppm
1
2
3
4
5
6
7
8
9
1.074
1.527
0.573
0.591
0.997
1.010
1.000
2.247
2.962
3.075
1.099
1.071
3.069
3.166

INSTRUMENT : PERKIN ELMER 1600 Series FTIR

Sample ID : Cefuroxime Axetil





126

Sample Name: CEFUROXIME AXETIL.

OBJECTIVE: - ISOLATION OF IMPURITY AT RRT ~1.60

The impurity at RRT-1.60 was isolated from Cefuroxime Axetil using preparative chromatography.

Enrichment Of Impurity

The enrichment of the impurity in sample was carried out using Cefuroxime Axetil. 1gm sample was dissolved in 100ml water: methanol (1:1 v/v) and kept in sunlight for about 8 hours hrs to enrich the impurity. This solution was filtered, lyophilized and loaded on Preparative LC for isolation.

Preparative HPLC

Column

- Type: Spherisorb C_{18}
- Dimensions: 250 mm x 20 mm
- Particle size: 5µm
- Temperature: Ambient

Detector setting

- Wavelength: 280 nm

Mobile Phase

- Buffer: 0.1 M Ammonium acetate
- Mobile A: Buffer: Methanol (70: 30 v/v)
- Mobile B: Buffer: Methanol (40: 60 v/v)
- pH: As such

Sample preparation

40 mg lyophilized sample was dissolved in 8 ml water: methanol (1:1 v/v) and loaded on to preparative column.

Loading amount: 40 mg sample/loading

Chromatographic condition

S.No.	Time	Event	Value	Flow ml/min
1	0.01	B.conc	0.00	15.00
2	15.00	B.conc	100.0	15.00
3	25.00	B.conc	100.0	15.00
4	30.00	B.conc	0.00	15.00
5	35.00	B.conc	0.00	15.00

Fractions collected were monitored using the analytical method. Fractions having the impurity sample in (~95%) purity were concentrated using rotary evaporator at bath temperature 35°C under vacuum to distill out methanol. The combined fraction was then subjected to extraction with dichloromethane. The dichloromethane layer was fully evaporated using rotavapor at 35°C to obtain solid material (Purity ≥95% By HPLC area normalization method) as white solid.

SPECTRAL ANALYSIS

NMR SPECTRA

The NMR spectra of impurity at RRT – 1.60 were recorded on a 200 MHz Bruker DRX-200 instrument using $CDCl_3$ as solvent.

1H NMR

7.57 (s, 1H); 7.39 (d, 2H); 7.12 (d, 1H); 6.53 (d, 1H); 5.97 (m, 1H); 5.12 (s, 2H); 4.8 (m-2H); 4.12 (s, 3H); 3.6-3.3 (dd, 2H); 2.1 (s, 3H); 1.57 (d, 3H) ppm/δ

^{13}C NMR

169.4; 164.4; 161.9; 159.4; 156.5; 144.5; 142.7; 139.1; 128.4; 124.9; 119.8; 111.7; 88.8; 64.4; 63.5; 59.3; 57.6; 26.5; 20.9; 19.5 ppm/δ

Analysis of C13 and DEPT-135 carbon NMR spectrum indicates the presence of 20 C atoms and 2 CH_2 groups.

IR in KBr

The IR spectrum was recorded on FTIR – 8201 PC Shimadzu instrument using KBr pallet.

3477.9; 3362.4; 2942.0; 1784.5; 1731.7; 1682.1; cm^{-1}

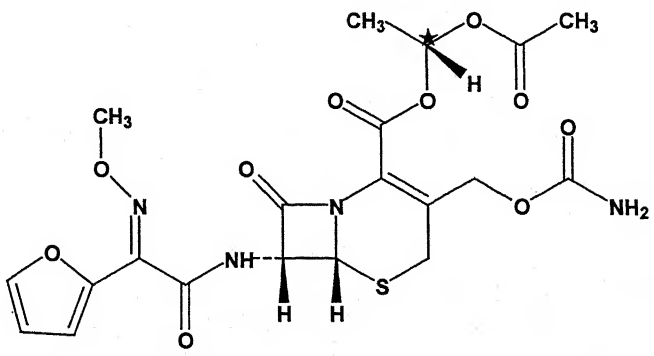
MASS SPECTRA

The compound exhibited a quasi-molecular ion peak at 528 implying a molecular weight of 510. For mass spectrum the sample was dissolved in methanol and spectrum was taken using Atmospheric Pressure Ionization technique having turbolon ionization source. The declustering potential was set at 6v; focusing potential at 60v, and ionization volt energy (IVE) 4000 at 350°C

Molecular weight	Structure
528.4	$(M+NH_4)^+$

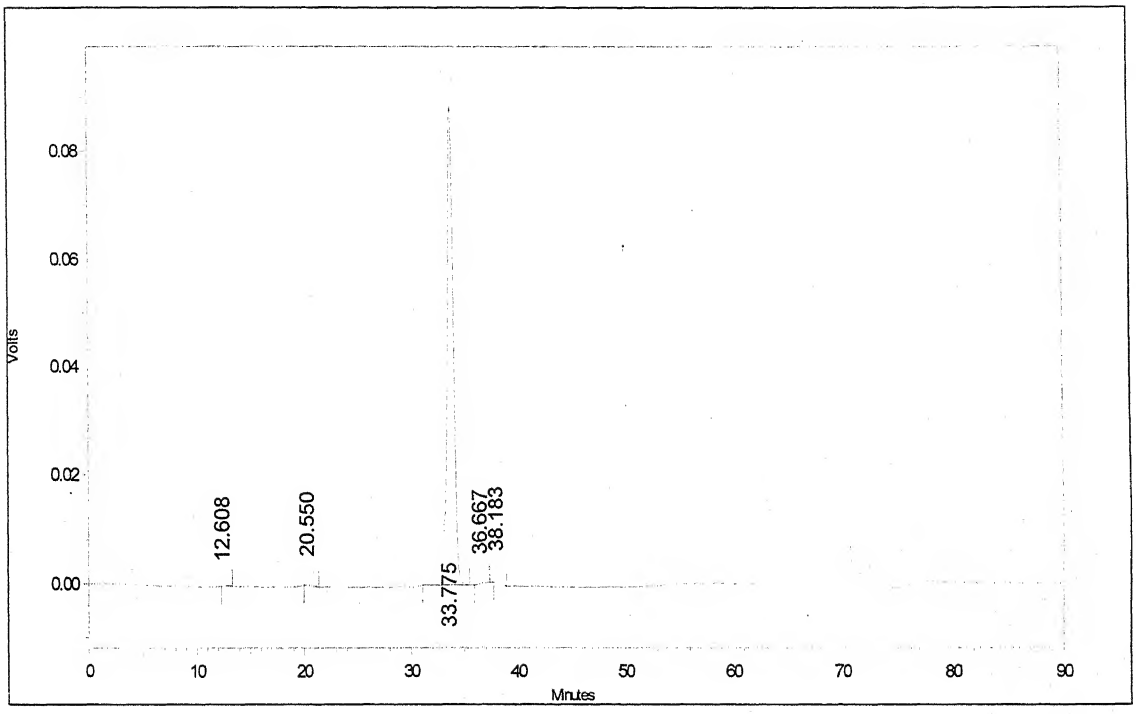
CONCLUSION

Spectroscopic (IR, NMR) analysis of the compound identified as Anti isomer-A of Cefuroxime Axetil,



{6-((2E)-3-aza-2-(2-furyl)-3-methoxyprop-2-enoylamino)(6R)-3-[(aminocarbonyloxy)methyl]-5-oxo-2H,6H,6aH-azetidino[2,1-b] 1,3-thiazin-4-ylcarbonyloxy}ethyl acetate.
(Anti isomer-A of Cefuroxime Axetil)

Method Name: C:\HPLC-23\Method\Cef.Axetil\Cef Axetil gradient.met
File Name : C:\HPLC-23\Data\Feb03\Cef. Axetil\23-020503.04
Aquired Time: 2/5/2003 1:53:16 PM
Sample ID: Anti Isomer A



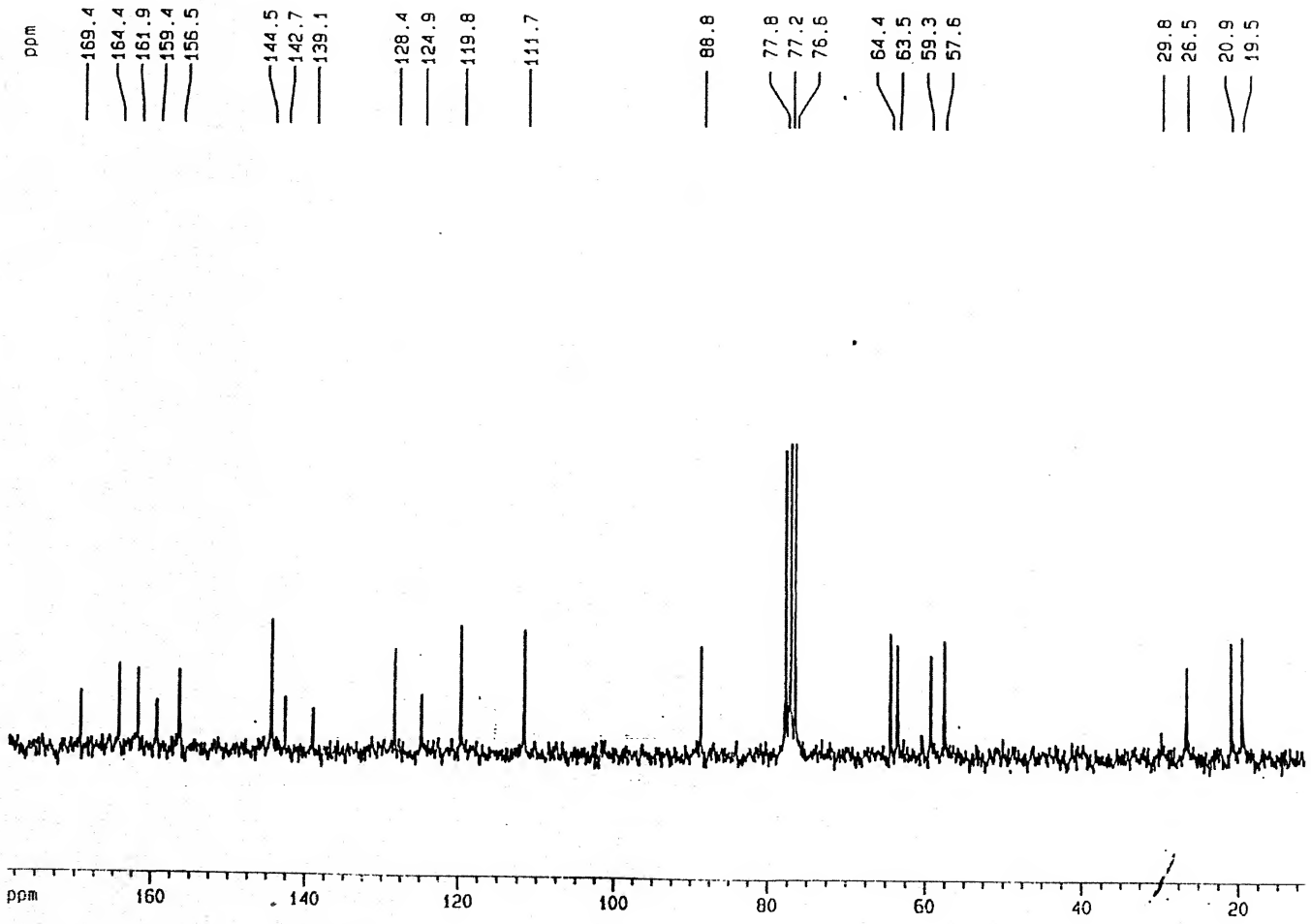
Detector A (278nm)

Pk #	Retention Time	Area	Area Percent	Name
1	12.608	17911	0.44	
2	20.550	54606	1.34	
3	33.775	3879458	95.20	**Anti Isomer A**
4	36.667	49455	1.21	
5	38.183	73841	1.81	

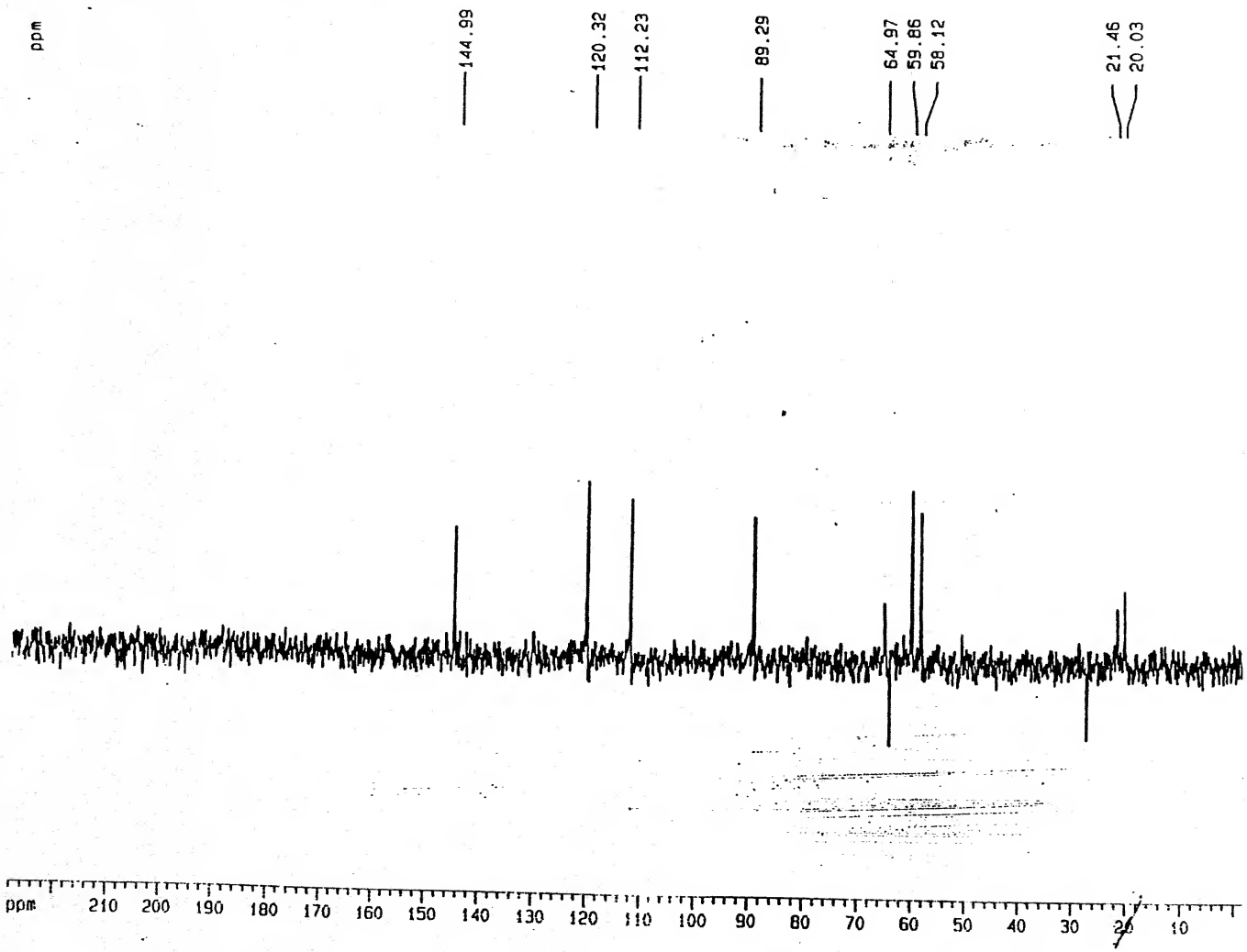
Totals		4075271	100.00	

Cefuroxime Axetil-Anti Isomer-A in CDCL3

CEFUROXIME AXETIL ANTI ISOMER (A) IN COCL3

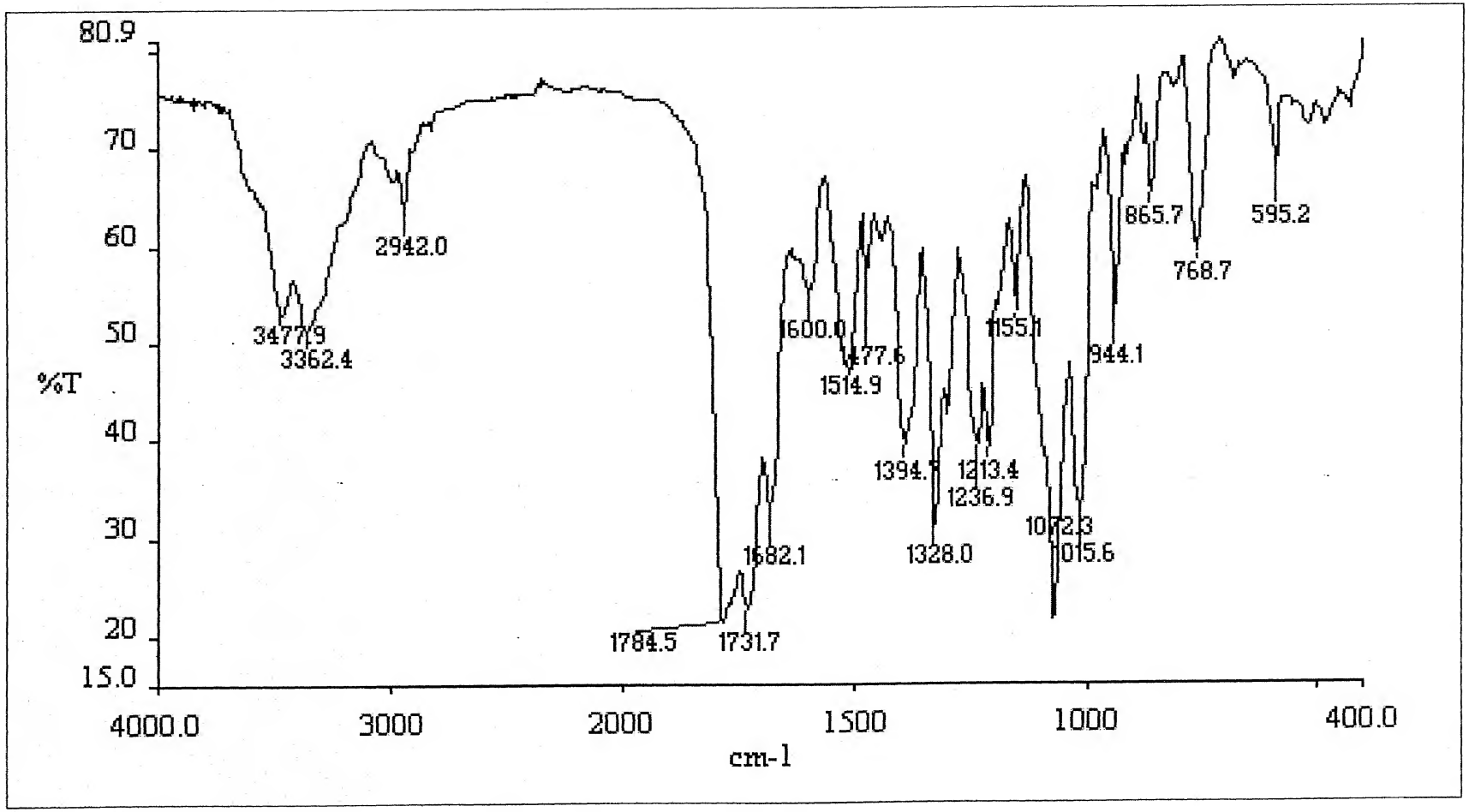


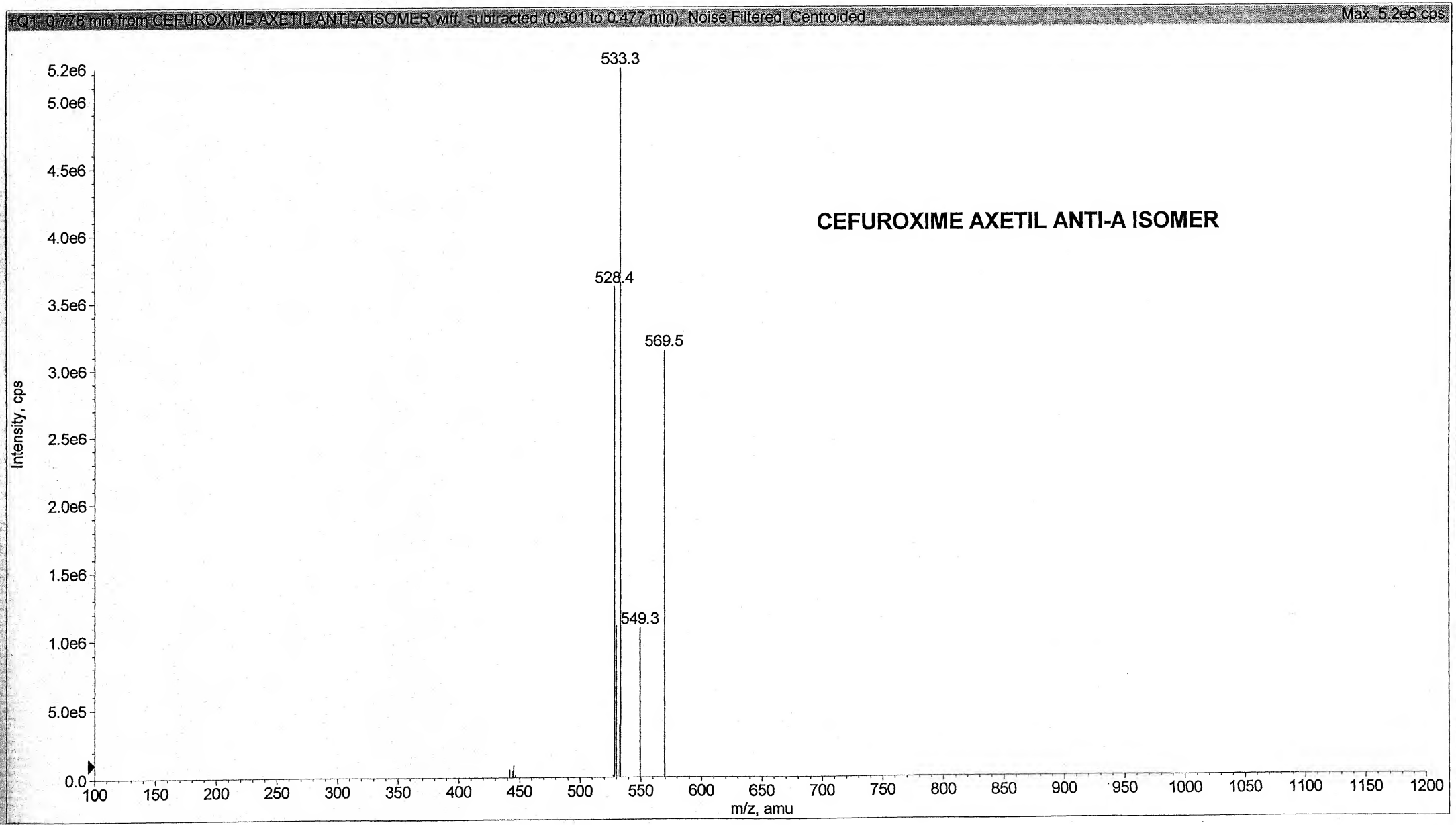
CEFUROXIME AXETIL ANTI ISOMER (A) IN CDCL3



INSTRUMENT : PERKIN ELMER 1600 Series FTIR

Sample ID : Cefuroxime Axetil Anti Isomer A





136

Sample Name: CEFUROXIME AXETIL ANTI-A ISOMER

OBJECTIVE: - ISOLATION OF IMPURITY AT RRT~1.82

The impurity at RRT-1.82 was isolated from Cefuroxime Axetil using preparative chromatography.

Enrichment Of Impurity

The enrichment of the impurity in sample was carried out using Cefuroxime Axetil Amorphous. 1gm sample was dissolved in 100ml water: methanol (1:1 v/v) and kept in sunlight for about 8 hours hrs to enrich the impurity. This solution was filtered, lyophilized and loaded on Preparative LC for isolation.

Preparative HPLC

Column

- Type: Spherisorb C_{18}
- Dimensions: 250 mm x 20 mm
- Particle size: 5µm
- Temperature: Ambient

Detector setting

- Wavelength: 280 nm

Mobile Phase

- Buffer: 0.1 M Ammonium acetate
- Mobile A: Buffer: Methanol (70: 30 v/v)
- Mobile B: Buffer: Methanol (40: 60 v/v)
- pH: As such

Sample preparation

40 mg lyophilized sample was dissolved in 8ml water: methanol (1:1 v/v) and loaded on to preparative column.

Loading amount: 40 mg sample/loading

Chromatographic condition

S.No.	Time	Event	Value	Flow ml/min
1	0.01	B.conc	0.00	15.00
2	15.00	B.conc	100.0	15.00
3	25.00	B.conc	100.0	15.00
4	30.00	B.conc	0.00	15.00
5	35.00	B.conc	0.00	15.00

Fractions collected were monitored using the analytical method. Fractions having the impurity sample in (~96%) purity were concentrated using rotary evaporator at bath temperature 35°C under vacuum to distill out methanol. The combined fraction was then subjected to extraction with dichloromethane. The dichloromethane layer was fully evaporated using rotavapor at 35°C to obtain solid material (Purity ≥96% By HPLC area normalization method) as white solid.

SPECTRAL ANALYSIS

NMR SPECTRA

The NMR spectra of impurity at RRT – 1.82 were recorded on a 200 MHz Bruker DRX-200 instrument using DMSO as solvent.

1H NMR

9.63 (d, 1H); 7.84 (d, 1H); 7.25 (d, 1H); 6.92 (d, 1H); 6.68 (dd, 2H); 5.86-5.80 (q, 1H); 5.22 (d, 2H); 4.79 (dd, 2H); 3.99 (s, 3H); 3.6-3.3 (dd, 2H); 2.0 (s, 3H); 1.46 (d, 3H) ppm/δ

Proton signal (H of Furan ring) at 6.8 is shifted downfield to 7.25 indicates E-Isomer of Cefuroxime axetil.

^{13}C NMR

168.88; 164.32; 161.6; 159.2; 156.12; 144.15; 142.40; 138.8; 126.98; 124.75; 119.46; 111.36; 89.65; 64.12; 63.01; 59.18; 57.15; 26.34; 20.69; 18.90 ppm/δ

Analysis of C13 and DEPT 135 carbon NMR spectrum indicates the presence of 20 C atoms and 2 CH2 groups.

IR in KBr

The IR spectrum was recorded on FTIR – 8201 PC Shimadzu instrument using KBr pallet.

3479.3; 3340.6; 2942.6; 1786.0; 1720.0; 1698.0; 1519.1; 1074.7 cm^{-1}

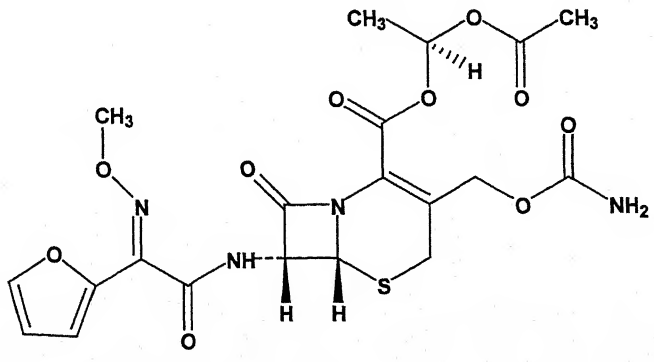
MASS SPECTRA

The compound exhibited a quasi-molecular ion peak at 528 implying a molecular weight of 510. For mass spectrum the sample was dissolved in methanol and spectrum was taken using Atmospheric Pressure Ionization technique having turbolon ionization source. The declustering potential was set at 6v; focusing potential at 60v, and ionization volt energy (IVE) 4000 at 350°C.

Molecular weight	Structure
528.5	$(M+NH_4^+)$

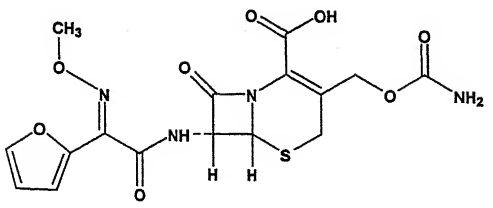
CONCLUSION

Spectroscopic (IR, NMR) analysis of the compound identified as Anti isomer-B of Cefuroxime Axetil,



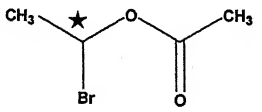
{6-((2E)-3-aza-2-(2-furyl)-3-methoxyprop-2-enoylamino)(6R)-3-[(aminocarbonyloxy)methyl]-5-oxo-2H,6H,6aH-azetidino[2,1-b] 1,3-thiazin-4-ylcarbonyloxy}ethyl acetate.
(Anti-B isomer of Cefuroxime axetil)

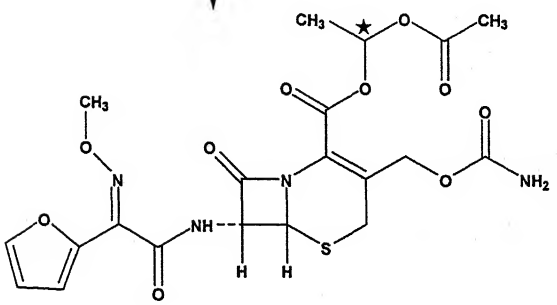
Proposed Formation Pathway Of Anti Isomer A & B



ANTI CEFUROXIME ACID (E-ISOMER)

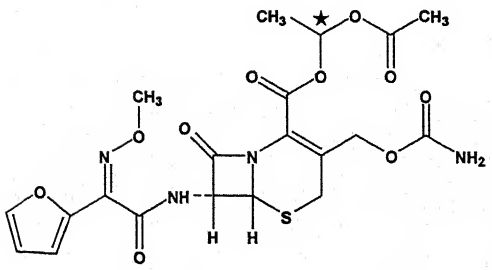
Condensation





CEFUROXIME AXETIL ANTI ISOMERS A & B

H^+ / SUN LIGHT



CEFUROXIME AXETIL

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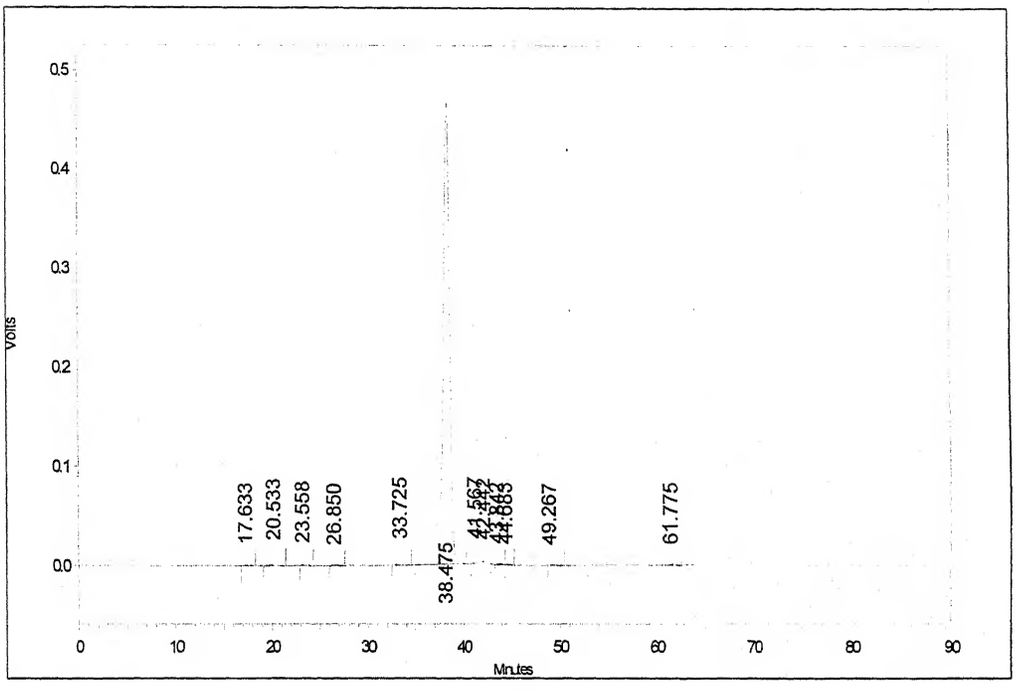
Aquired Time: 2/5/2003 3:32:57 PM
Sample ID: Anti Isomer B



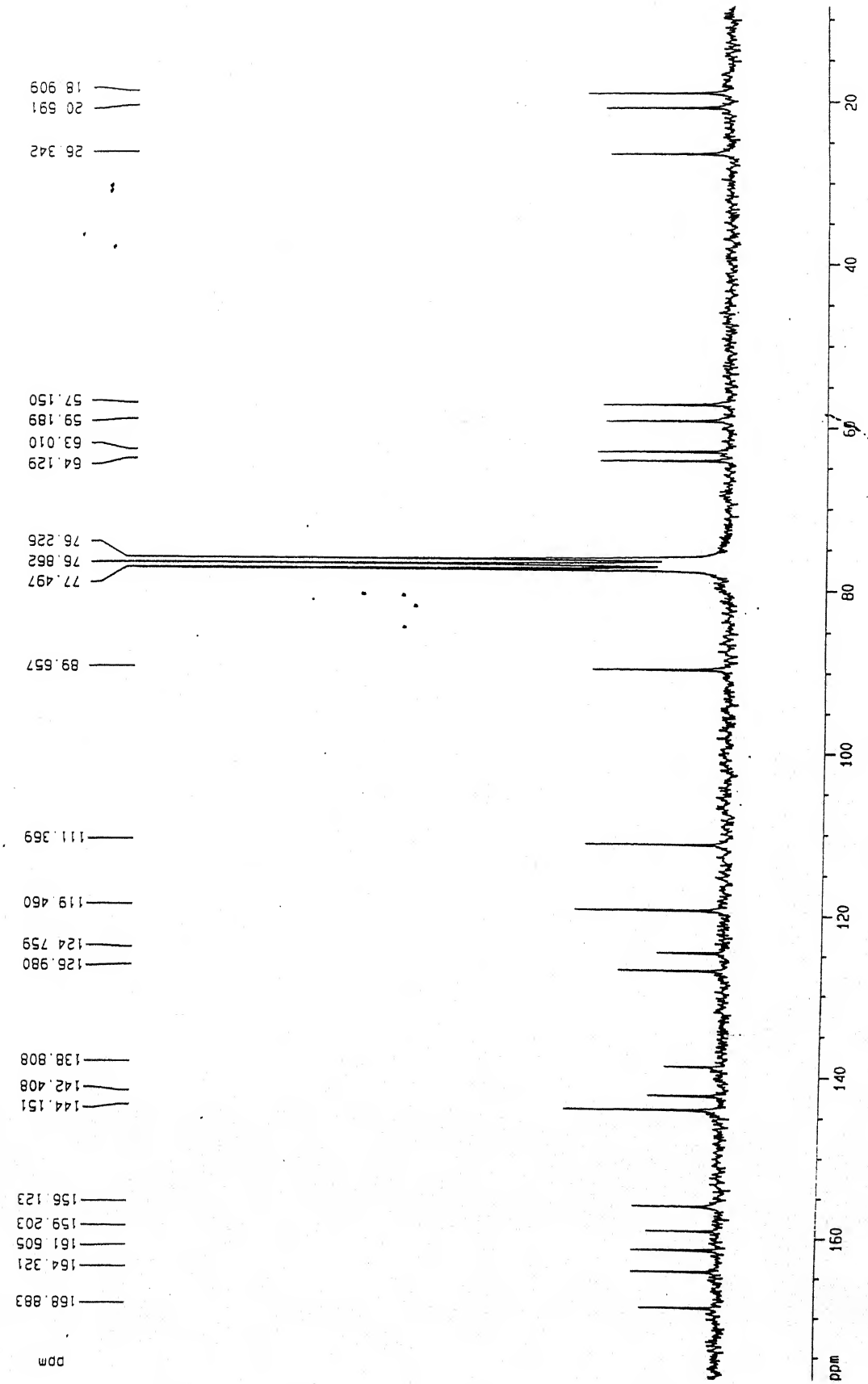
Detector A (278nm)

Pk #	Retention Time	Area	Area Percent	Name
1	17.633	37090	0.16	
2	20.533	193082	0.82	
3	23.558	77936	0.33	
4	26.850	21666	0.09	
5	33.725	241394	1.02	
6	38.475	22739299	96.21	**Anti Isomer B**
7	41.567	123474	0.52	
8	42.442	87204	0.37	
9	43.842	24893	0.11	
10	44.683	15252	0.06	
11	49.267	20336	0.09	
12	61.775	53209	0.23	

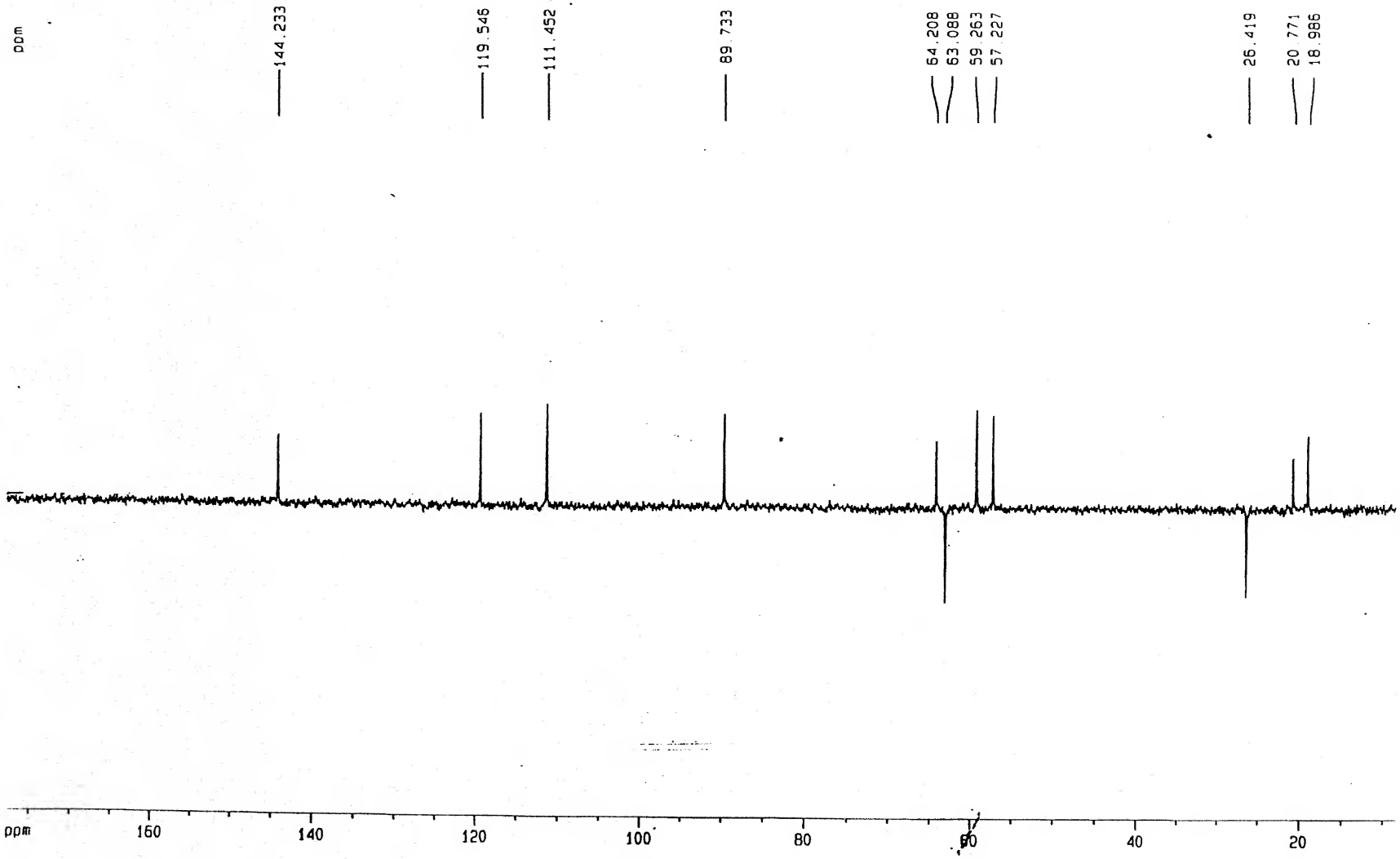
Totals		23634835	100.00	

Cefuroxime Axetil:Anti Isomer-B in DMSO-d6

CEFUROXIME AXETIL ANTI ISOMER (B) IN DMSO

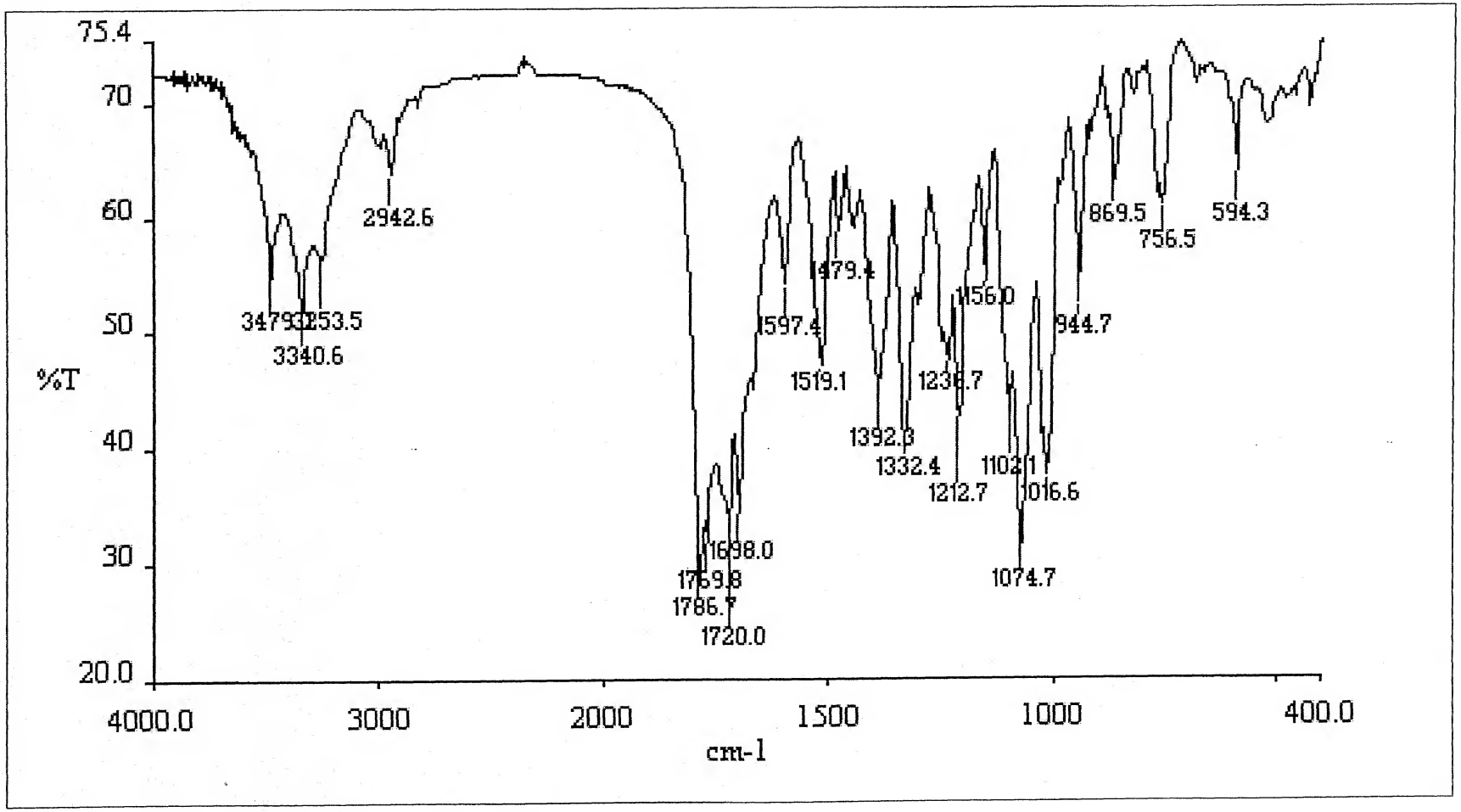


CEFUROXIME AXETIL ANTI ISOMER (B) IN DMSO

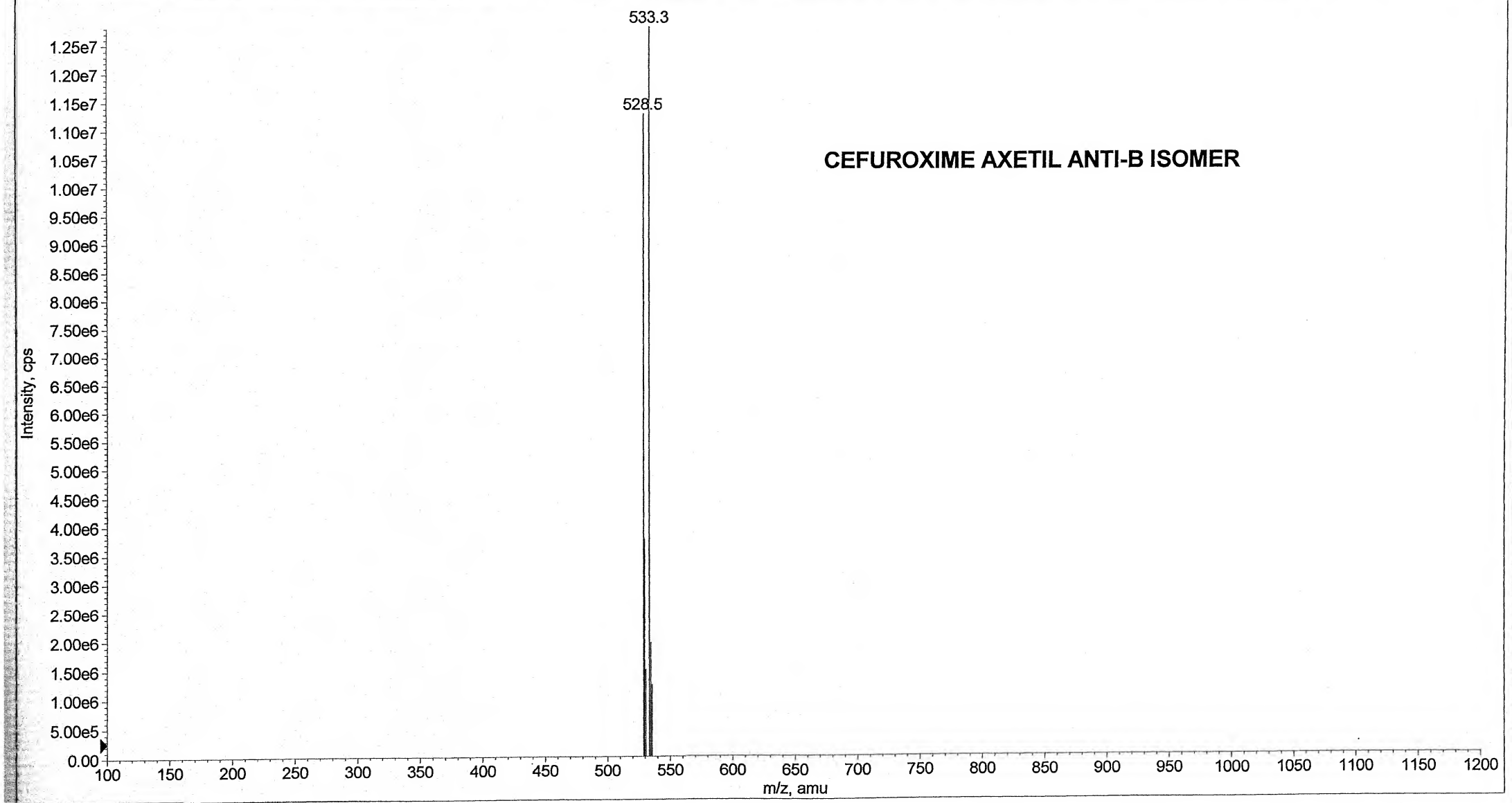


INSTRUMENT : PERKIN ELMER 1600 Series FTIR

Sample ID : Cefuroxime Axetil Anti Isomer B



+Q1: 0.452 to 0.477 min from CEFUROXIME AXETIL ANTI-B ISOMER.wiff, subtracted (0.000 to 0.226 min), Noise Filtered, Centroided Max. 1.3e7 cps.



147

Sample Name: CEFUROXIME AXETIL ANTI-B ISOMER

OBJECTIVE: - ISOLATION OF IMPURITY AT RRT~ 1.25

The impurity at RRT-1.25 was isolated from Cefuroxime Axetil Amorphous using preparative chromatography.

Enrichment Of Impurity

The enrichment of the impurity in sample was carried out using Cefuroxime Axetil Amorphous. 1gm sample was dissolved in 100ml water: methanol (1:1 v/v) and kept in oven at 80oC for 12 hrs to enrich the impurity. This solution was filtered, lyophilized and loaded on Preparative LC for isolation.

Preparative HPLC

Column

- Type: Spherisorb C_{18}
- Dimensions: 250 mm x 20 mm
- Particle size: 5µm
- Temperature: Ambient

Detector setting

- Wavelength: 280 nm

Mobile Phase

- Buffer: 0.1 M Ammonium acetate
- Mobile A: Buffer: Methanol (70: 30 v/v)
- Mobile B: Buffer: Methanol (40: 60 v/v)
- pH: As such

Sample preparation:

8ml sample was loaded on to preparative column.

Loading amount: 80 mg sample/loading

Chromatographic condition

S.No.	Time	Event	Value	Flow ml/min
1	0.01	B.conc	0.00	15.00
2	15.00	B.conc	100.0	15.00
3	25.00	B.conc	100.0	15.00
4	30.00	B.conc	0.00	15.00
5	35.00	B.conc	0.00	15.00

Fractions collected were monitored using the analytical method. Fractions having the impurity sample in (~95%) purity were concentrated using rotary evaporator at bath temperature 35°C under vacuum to distill out methanol. The combined fraction was then subjected to extraction with dichloromethane. The dichloromethane layer was fully evaporated using rotavapor at 35°C to obtain solid.

SPECTRAL ANALYSIS

NMR SPECTRA

The NMR spectra of impurity at RRT – 1.25 were recorded on a 200 MHz Bruker DRX-200 instrument using $CDCl_3$ as solvent.

1H NMR

7.50 (d, 1H); 7.45 (d, 1H); 6.91 (m, 2H); 6.48 (d, 2H); 5.83 (q, 1H); 5.38-5.30 (dd, 1H); 5.02 (b, 1H); 4.67 (b, 2H); 4.52 (s, 2H); 4.08 (s, 3H); 2.11 (s, 3H); 1.55 (m, 3H) ppm/δ

^{13}C NMR

169.2; 165.0; 163.6; 160.2; 156.0; 145.4; 144.7; 142.7; 122.4; 119.3; 114.7; 111.5; 89.7; 66.1; 64.3; 60.2; 53.1; 49.9; 20.7; 19.4. ppm/δ

Analysis of C13 NMR spectrum indicates the presence of 20 C atoms.

IR in KBr

The IR spectrum was recorded on FTIR – 8201 PC Shimadzu instrument using KBr pallet.

3476.5; 3357.4; 2939.8; 1763.7; 1681.2; 1599.0; cm^{-1}

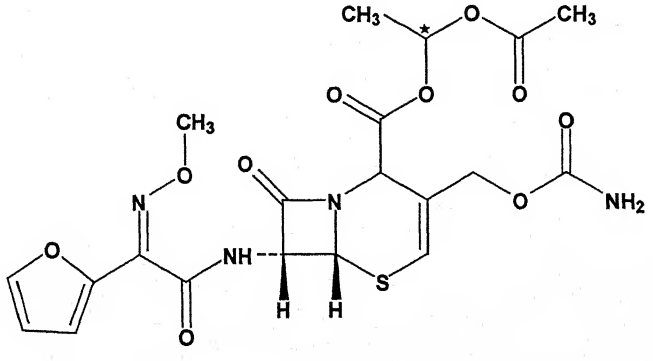
MASS SPECTRA

The compound exhibited a quasi-molecular ion peak at 528 implying a molecular weight of 510. For mass spectrum the sample was dissolved in methanol and spectrum was taken using Atmospheric Pressure Ionization technique having turbolon ionization source. The declustering potential was set at 6 v; focusing potential at 60v, and ionization volt energy (IVE) 4000 at 350°C

Molecular weight	Structure
528.4	$(M+NH_4^+)$

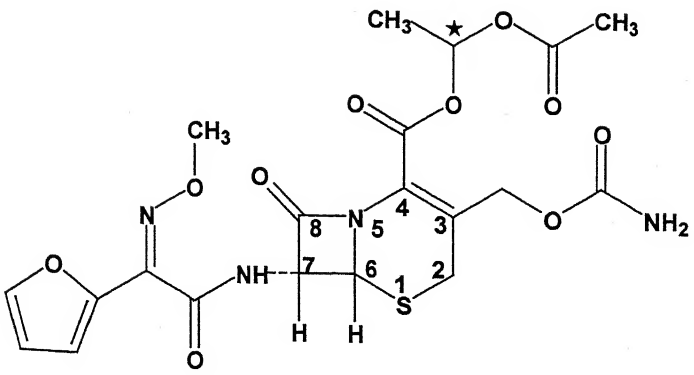
CONCLUSION

Spectroscopic (IR, NMR) analysis of the compound identified as Delta –2 Isomers of Cefuroxime Axetil,



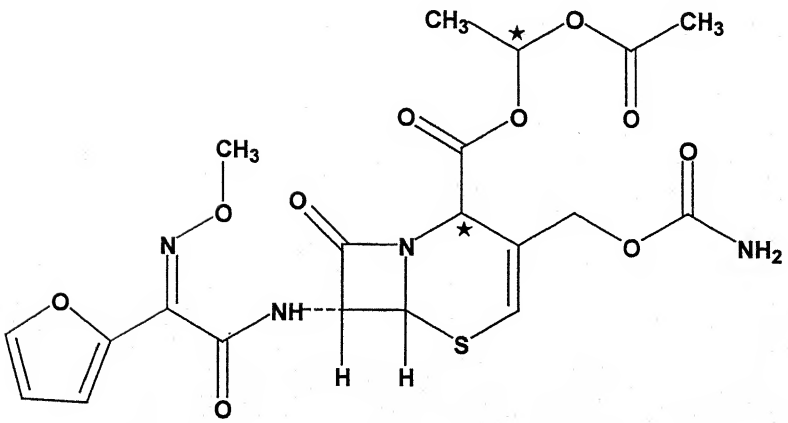
{(6R)-6-((2Z)-3-aza-2-(2-furyl)-3-methoxyprop-2-enoylamino)-3-[(aminocarbonyloxy)methyl]-5-oxo-4H,6H,6aH-azetidino[2,1-b] 1,3-thiazin-4-ylcarbonyloxy}ethyl acetate.
(Delta-2 isomer of cefuroxime axetil)

PROPOSED FORMATION PATHWAY OF Δ^2 ISOMER



CEFUROXIME AXETIL

Base /Δ^2



CEFUROXIME AXETIL - Δ^2 ISOMER

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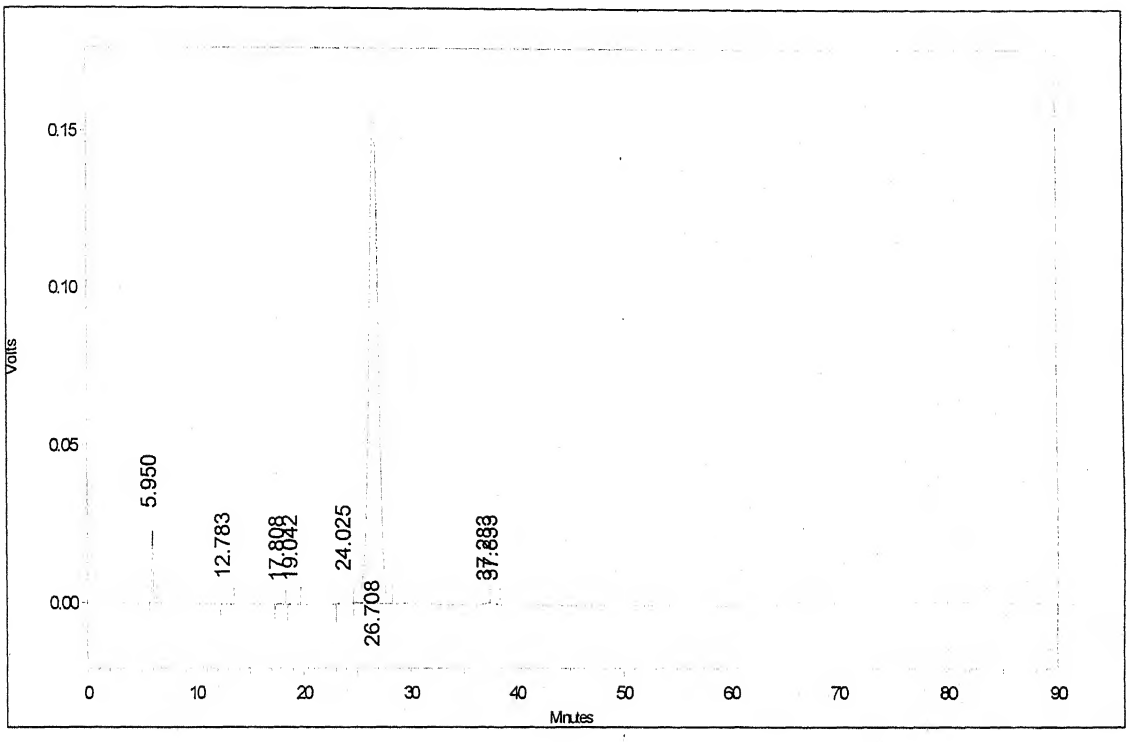
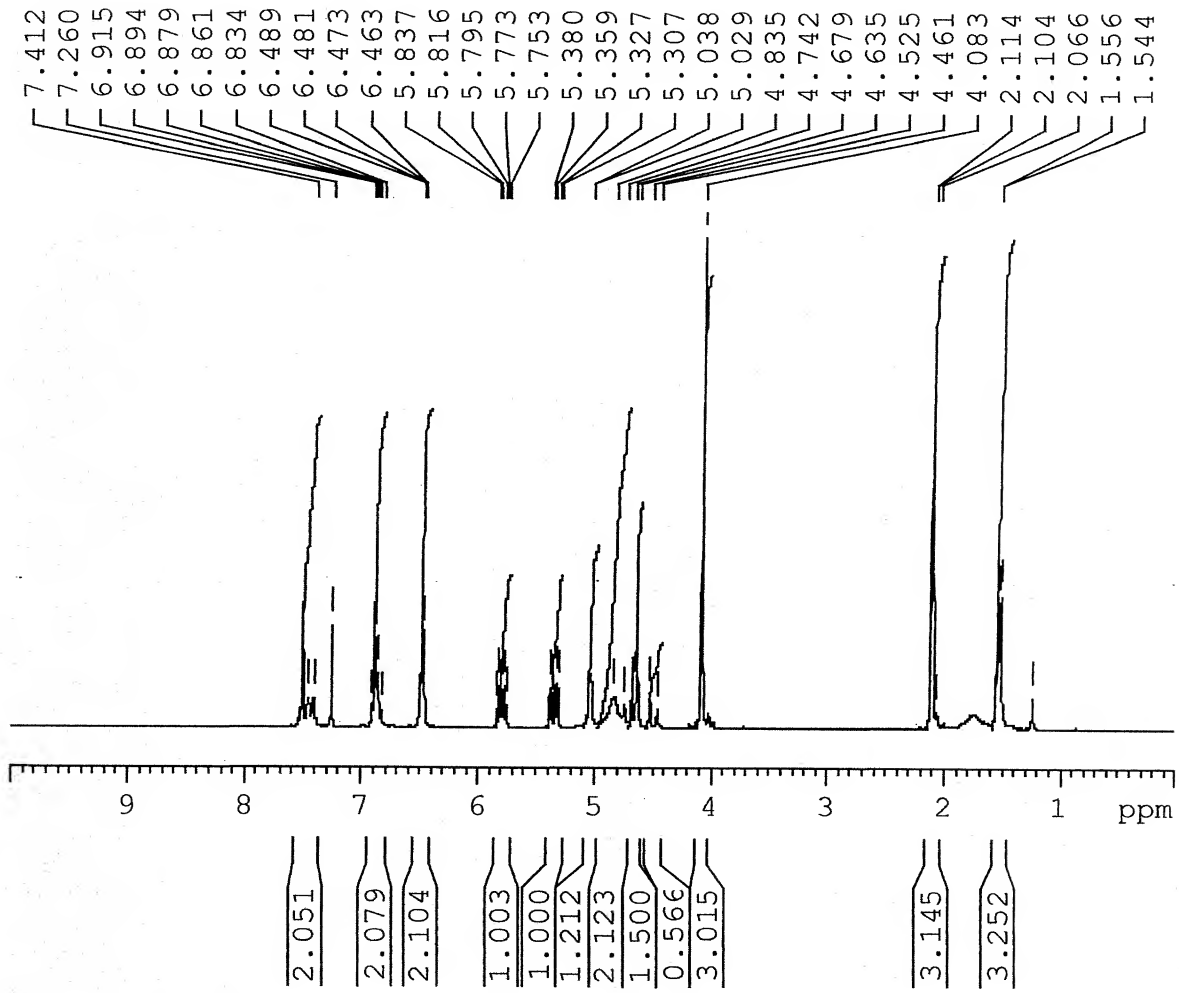
Sample ID: Delta Isomer



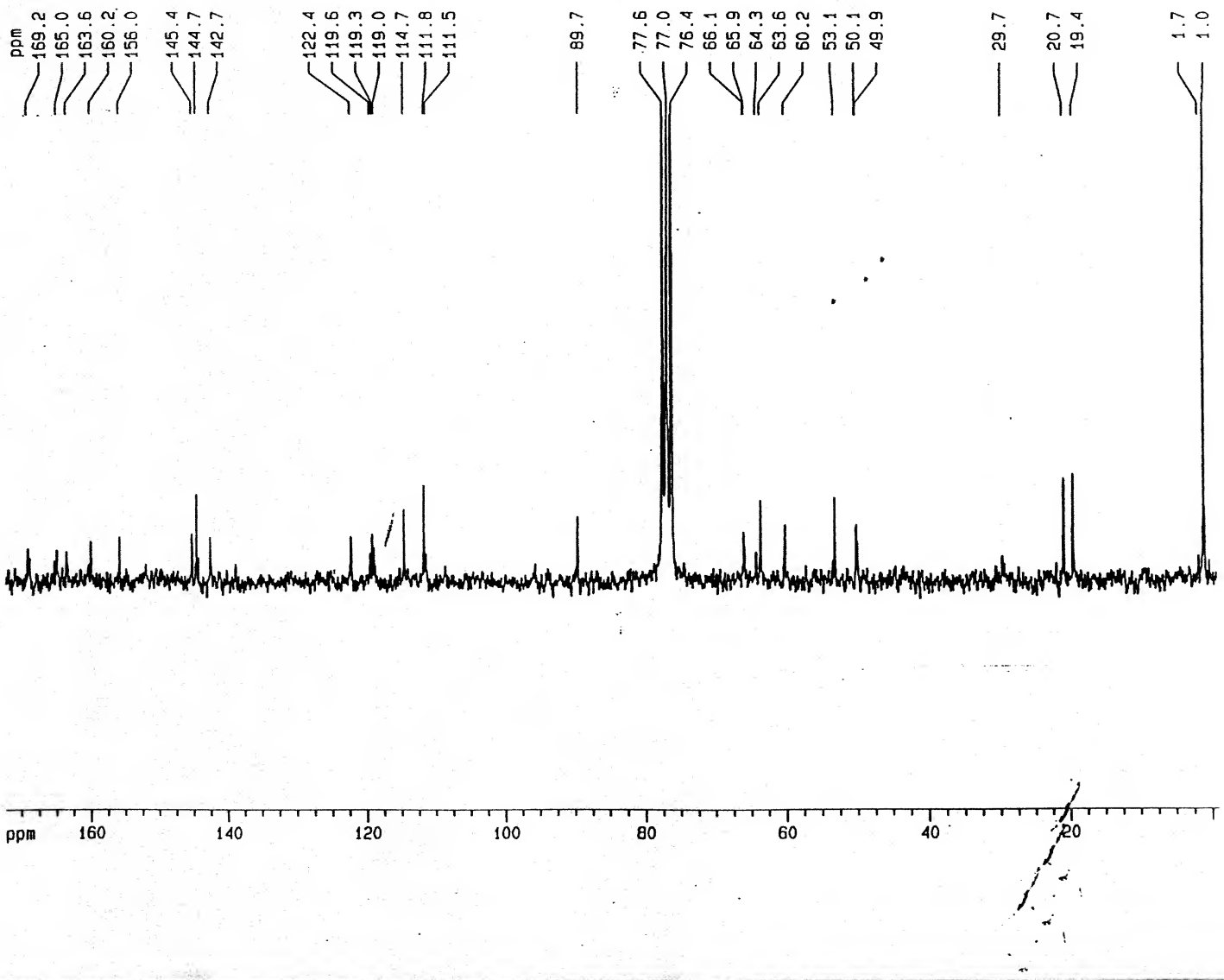
Detector A (278nm) Pk #	Retention Time	Area	Area Percent	Name
1	5.950	212309	2.11	
2	12.783	34270	0.34	
3	17.808	8735	0.09	
4	19.042	20133	0.20	
5	24.025	122754	1.22	
6	26.708	9660497	95.92	Delta Isomer
7	37.283	7604	0.08	
8	37.833	5040	0.05	

Totals				
		10071342	100.00	

Cefuroxime Axetil Delta Isomer in CDCL3

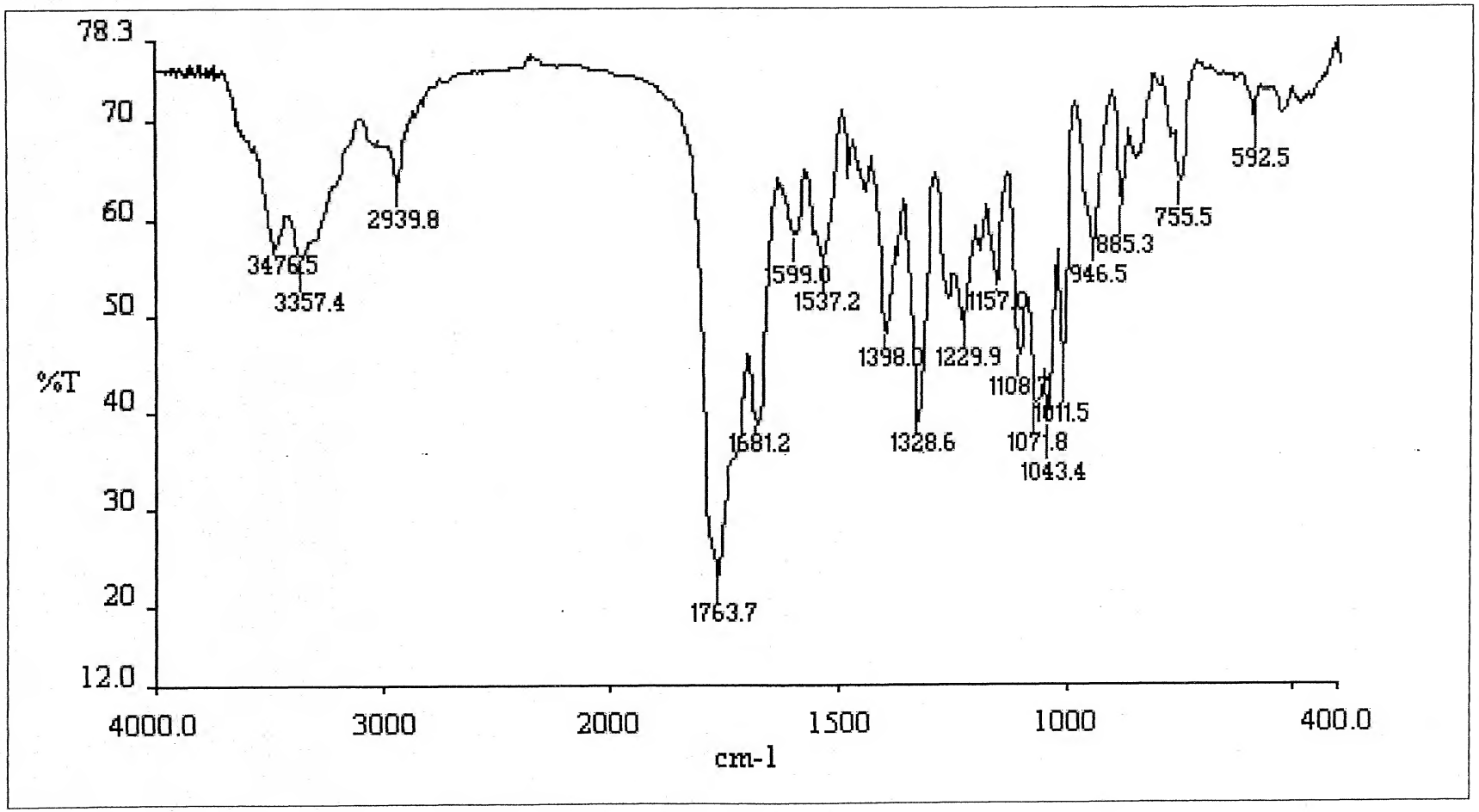


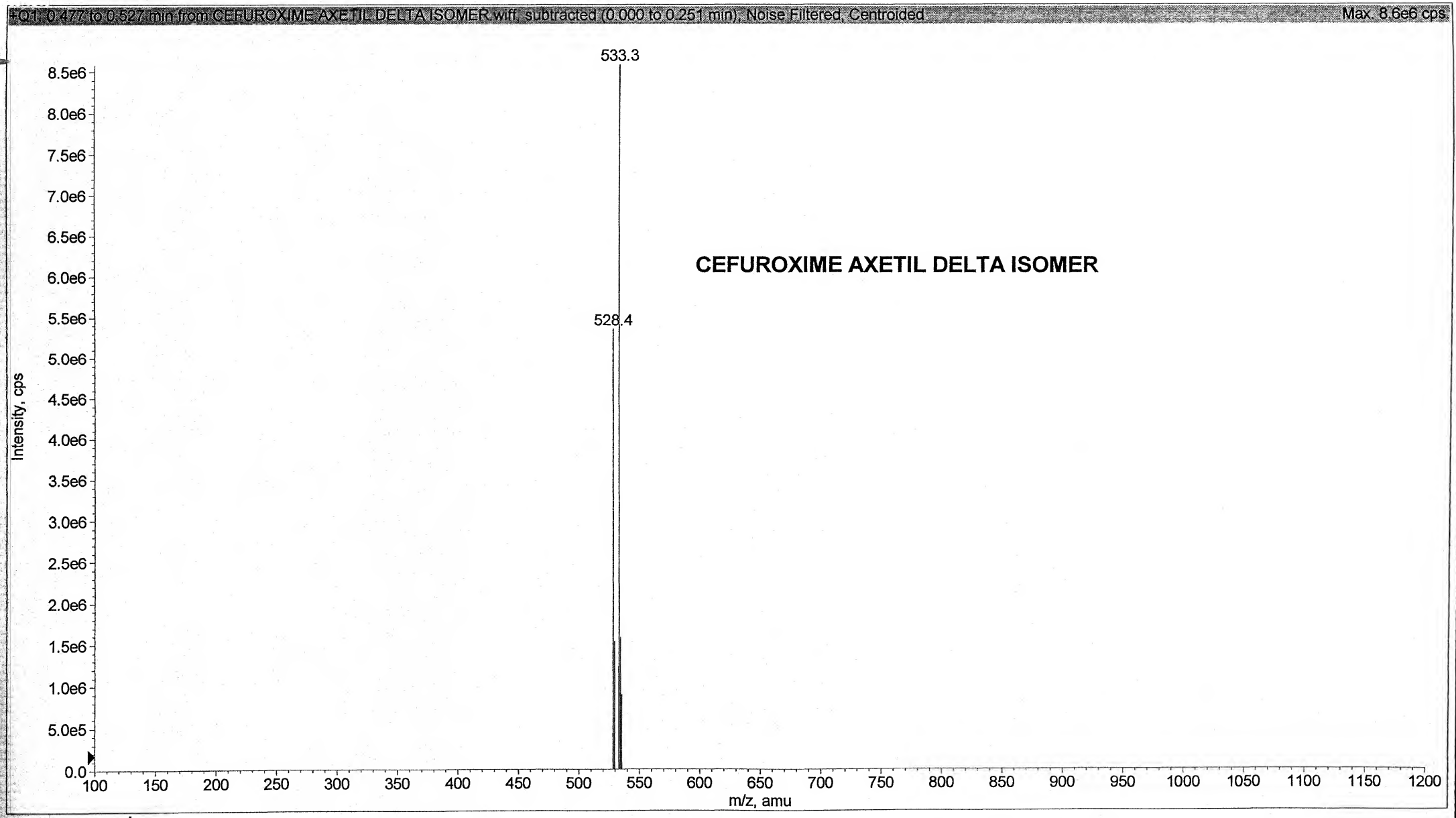
CTL/DELTA-2 ISOMER IN CDCL3



INSTRUMENT : PERKIN ELMER 1600 Series FTIR

Sample ID : Cefuroxime Axetil Delta Isomer





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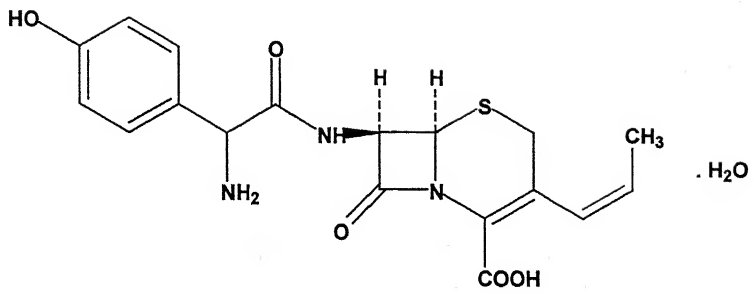
Sample Name: CEFUROXIME AXETIL DELTA ISOMER

Product: Cefprozil



Chemical name: - [6R-[6alpha,7beta(R*)]]-7-[[(Amino(4-hydroxy-phenyl) acetyll] amino]-8-oxo-3- (1-propenyl)-5-thia-1-azabicyclo[4.2.0] oct-2-ene-2-carboxylicacid monohydrate.

Brand: Cefzil

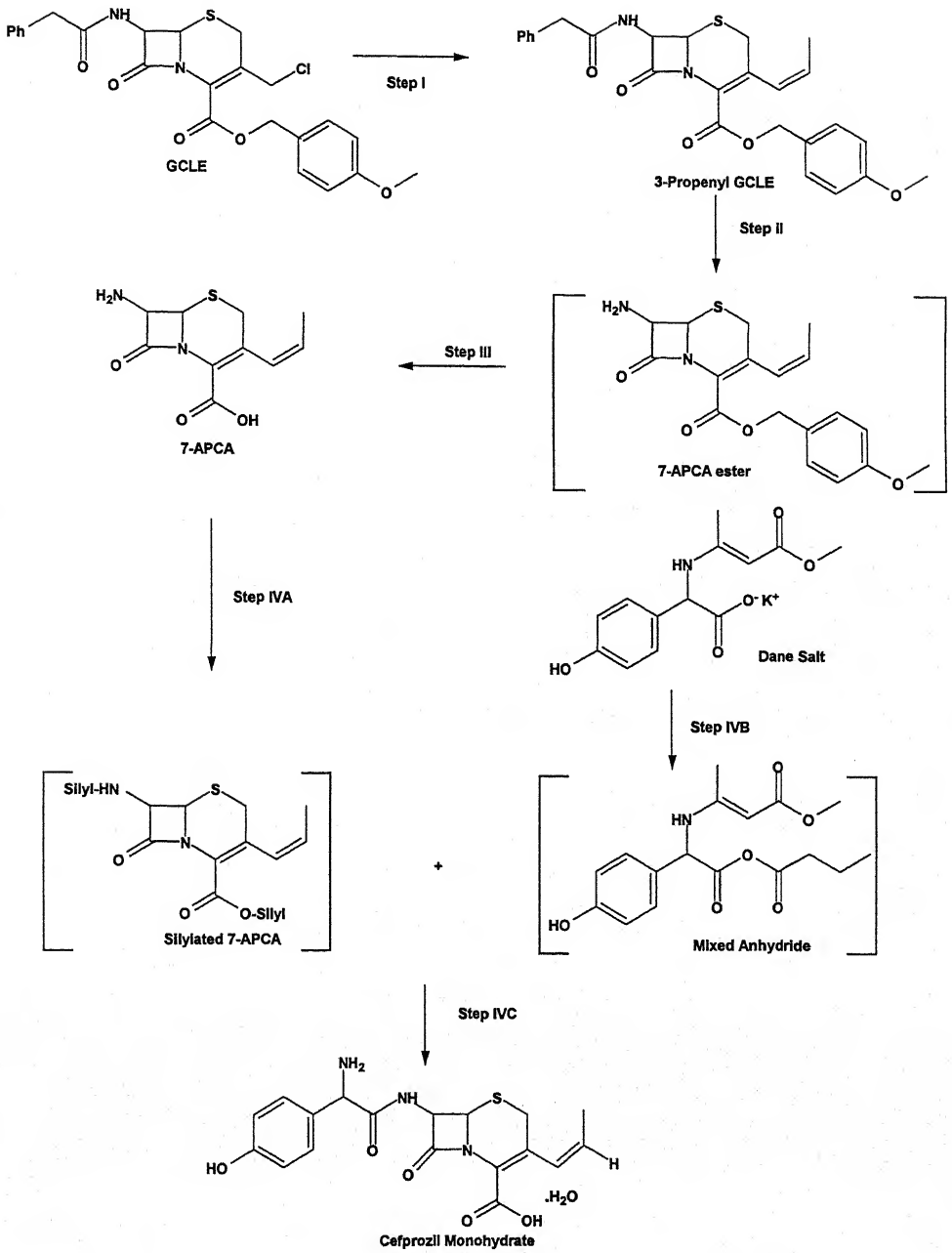
Molecular formula: $C_{18}H_{19}N_3O_5S.H_20$

Molecular Weight: 407.45

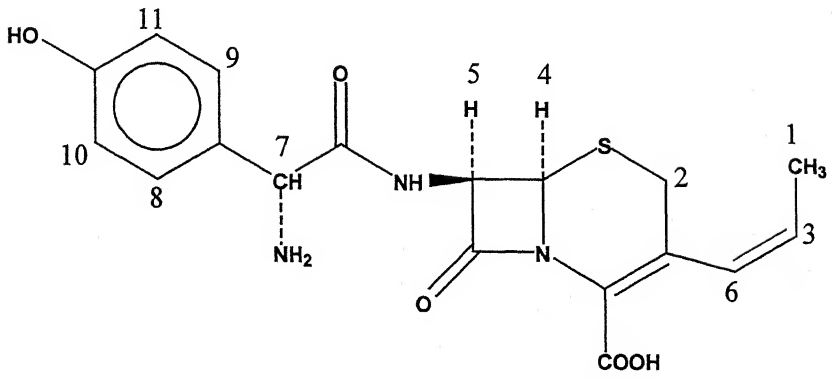
Uses: Antibacterial

Chemical Class: Second generation Beta-lactam antibiotics; Cephalosporin

Synthetic scheme of Cefprozil



CHARACTERIZATION OF CEFPROZIL



^{1}H NMR in D_2O

Cefprozil		Proton Assignment
δ (ppm)	Relative protons	
7.38	2	11&10
6.97	2	9&8
5.86	1	7
5.69	2	6&5
5.12	2	4&3
3.49-3.12	2	2
1.77	3	1

IR in KBr

Frequency cm^{-1}

Cefprozil	Assignment
Frequency cm^{-1}	
3540.6	-OH stretch
3050.2	-N-H stretch
1761.2	β-Lactam C=O
1682.6	CONH

Method Name : C:\HPLC-23\Method\Cefrozil\Cefprozil RS.met
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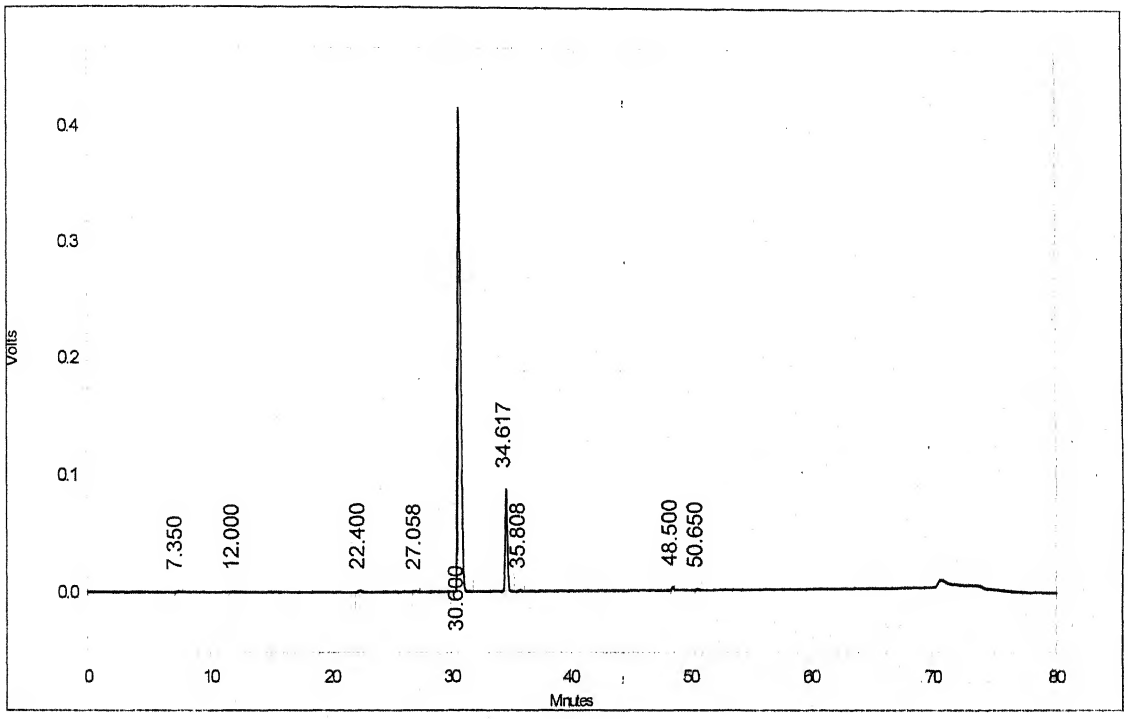
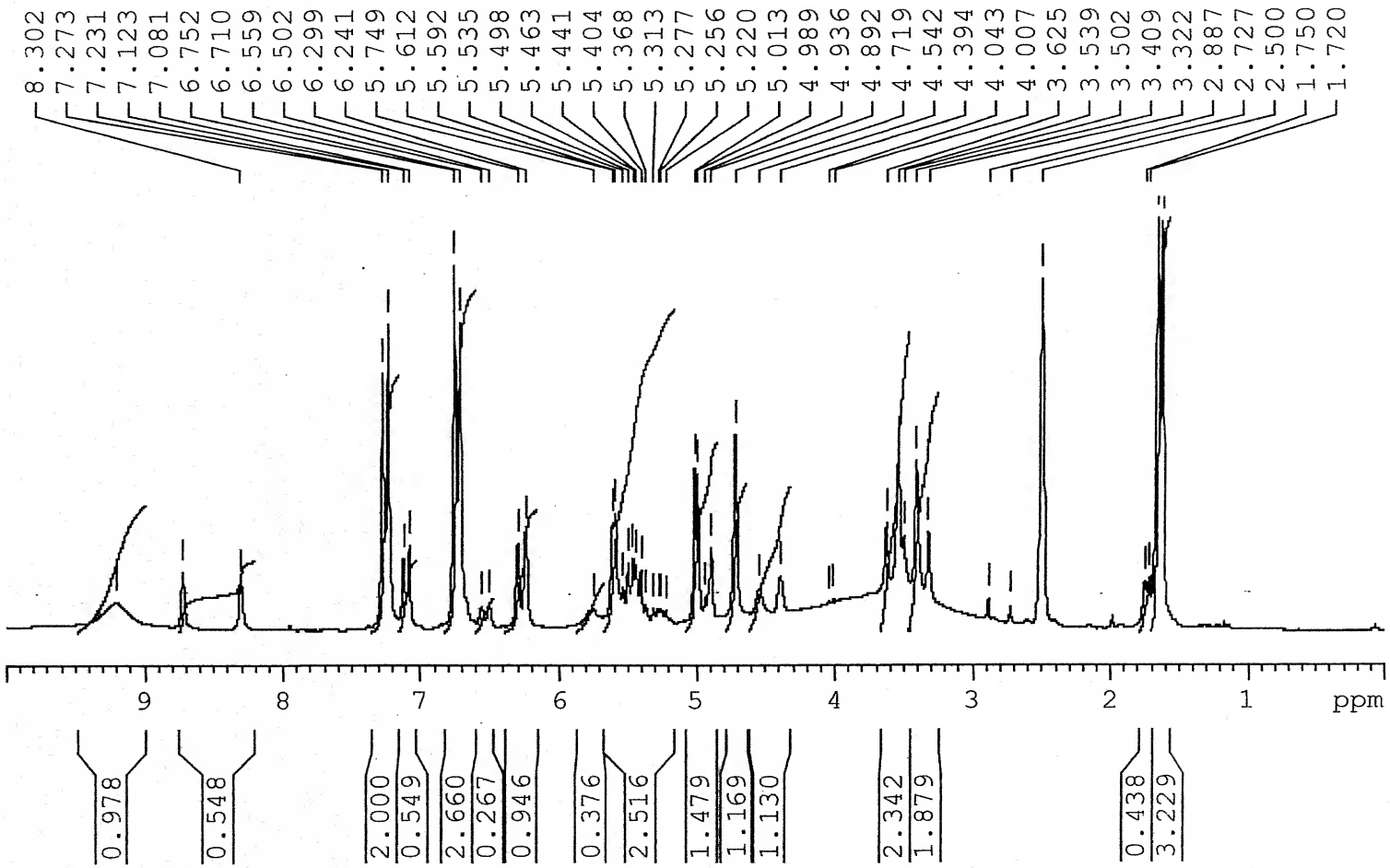
Aquired Time: 9/21/2003 3:07:35 PM
Sample ID : Cefprozil



Detector A (280nm)

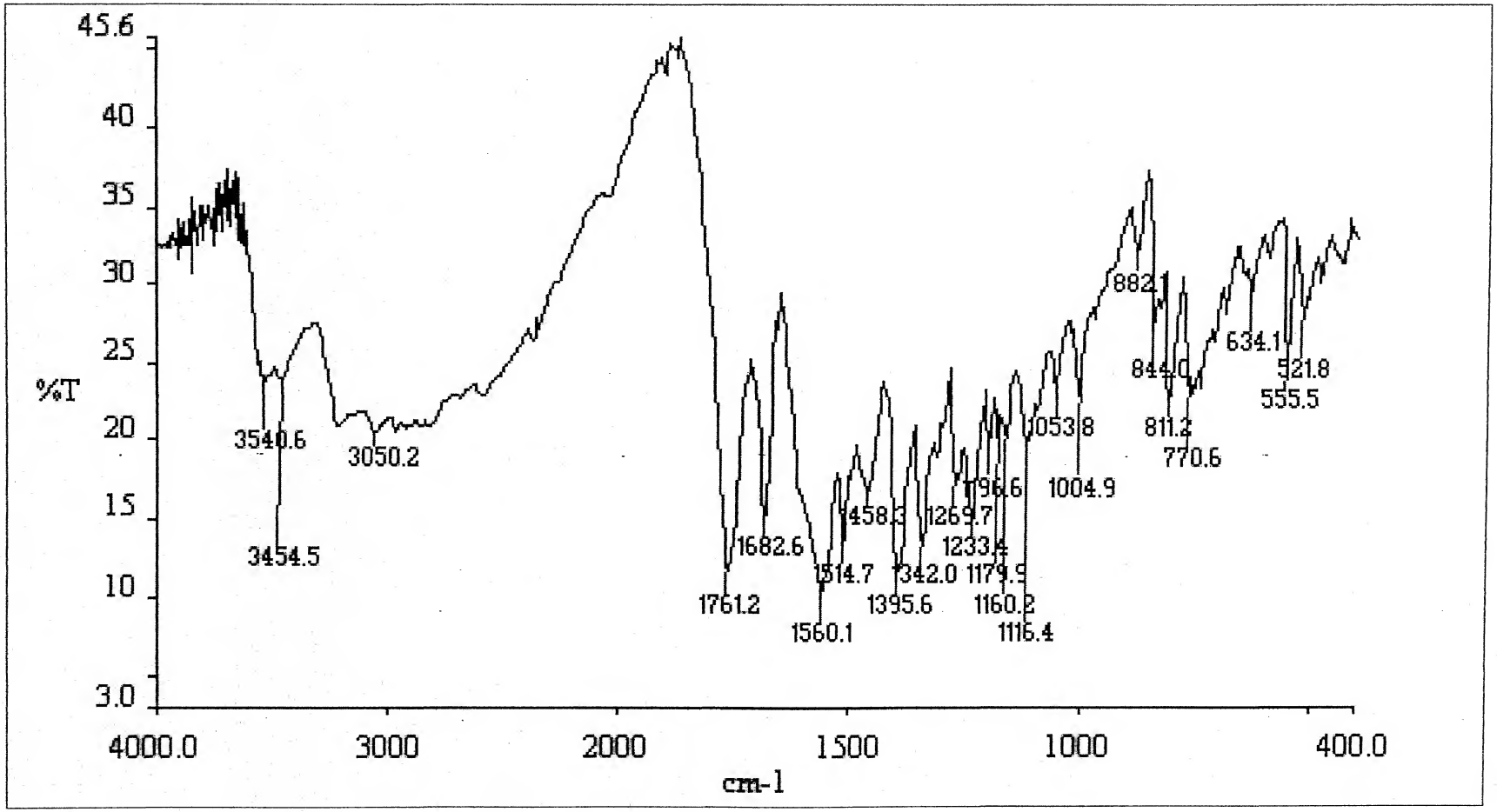
Pk #	Retention Time	Area	Area Percent	Relative RT	Name
1	7.350	4244	0.05	0.24	?
2	12.000	6914	0.08	0.39	?
3	22.400	15622	0.18	0.73	?
4	27.058	4512	0.05	0.88	?
5	30.600	7240386	85.29	1.00	Cefprozil (Z - Isomer)
6	34.617	1186514	13.98	1.13	Cefprozil (E - Isomer)
7	35.808	9737	0.11	1.17	?
8	48.500	15682	0.18	1.59	?
9	50.650	5709	0.07	1.66	?
Totals		8489320	100.00		

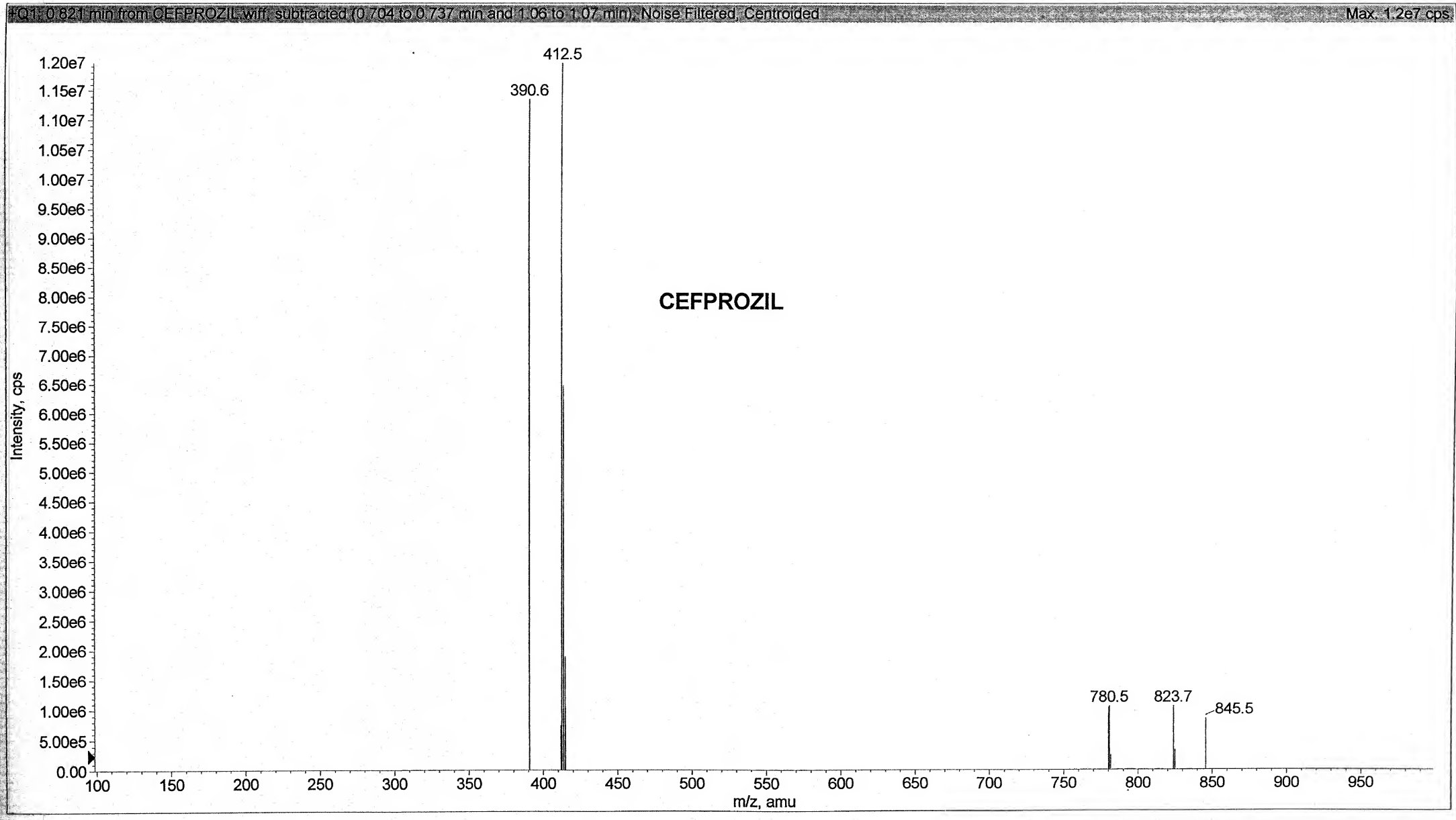
Cefprozil monohydrate in DMSO-d6



INSTRUMENT : PERKIN ELMER 1600 Series FTIR

Sample ID : Cefrozil monohydrate





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Sample Name: CEFPROZIL

Mass m/z amu

Cefprozil	Assignment
390.3	$(M+H)^+$
412.2	(M+Na)

OBJECTIVE: - ISOLATION OF IMPURITY AT RRT~ 1.59

This impurity at RRT ~ 1.59 was isolated cefprozil sample using preparative HPLC.

Preparative isolation

The chromatographic condition used was as follows:

Column

- Type: X-Terra C_{18}
- Dimensions: 300 mm x 19 mm
- Particle size: 7 µm

Detector setting

- Wavelength: 280 nm
- Flow rate: 15 ml/min

Mobile Phase

- Mobile A: Water (0.005% acetic Acid)
- Mobile B: Acetonitrile

Sample preparation

~100 mg of the enriched (~ 40%) sample dissolved in 8 ml distilled water.

Loading amount: 100 mg sample/injection

Chromatographic condition

S.No.	Time	Event	Value	Flow ml/min
1.	0.01	B.conc	0.00	15.0
2.	5.00	B.conc	0.00	15.0
3.	10.00	B.conc	10.00	15.0
4.	15.00	B.conc	20.00	15.0
5.	20.00	B.conc	40.00	15.0
6.	30.00	B.conc	70.00	15.0
7.	32.00	B.conc	100.00	15.0
8.	35.00	B.conc	100.00	15.0
9.	40.00	B.conc	0.00	15.0

Fractions collected were monitored using the analytical method. Fractions having the impurity sample in (~94%) purity were concentrated using rotary evaporator at bath temperature 35°C under vacuum to distill out acetonitrile. The combined fractions were then lyophilized to furnish ≥94 % (By HPLC area normalization method) pure material, as white solid.

The solid obtained was taken for further evaluation of the purity and also for various spectroscopic studies.

SPECTRAL ANALYSIS

NMR SPECTRA

The NMR spectra of impurity at RRT ~ 1.59 was recorded on a 200 MHz Bruker DRX-200 instrument using DMSO-d_6.

^{1}H NMR

8.8 (d, 1H); 7.41-7.37 (d, 2H); 7.25-7.21 (d, 2H); 6.39-6.33 (d, 1H); 5.75-5.71 (d, 1H); 5.41-5.32 (m, 1H); 4.55-4.51 (m, 1H); 4.46 (d, 1H); 4.26-4.19 (q, 2H); 3.56-3.51 (dd, 2H); 1.66-1.62 (d, 3H); 1.32-1.25 (t, 3H) ppm/δ

IR in KBr

The IR spectrum was recorded on FTIR-8201PC Shimadzu instrument using KBr pallet.

3210.1; 3019.8; 1758.9; 1691.0; 1558.3; 1222.8 cm^{-1}

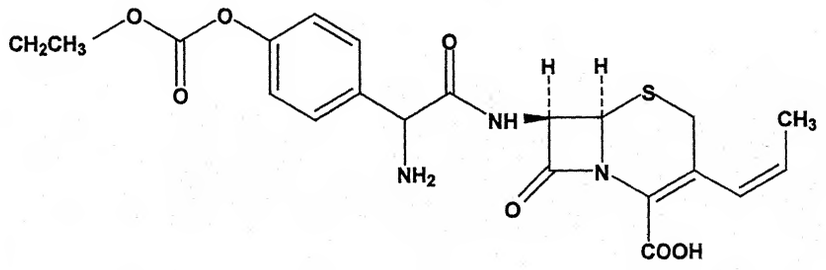
MASS SPECTRA

The compound exhibited a quasi-molecular ion peak at 462 implying a molecular weight of 461. The mass spectrum was recorded on PE SCIEX API-3000 triple quadruple mass spectrometer. For mass spectrum the sample was dissolved in methanol and spectrum was taken using API technique having turbolon ionization source. The declustering potential was set at 6 v; focusing potential at 60v, and ionization volt energy (IVE) 4000 at 350°C.

Molecular weight	Structure
462.2	$(M+H)^+$ Molecular ion
479.3	$M+NH_4$

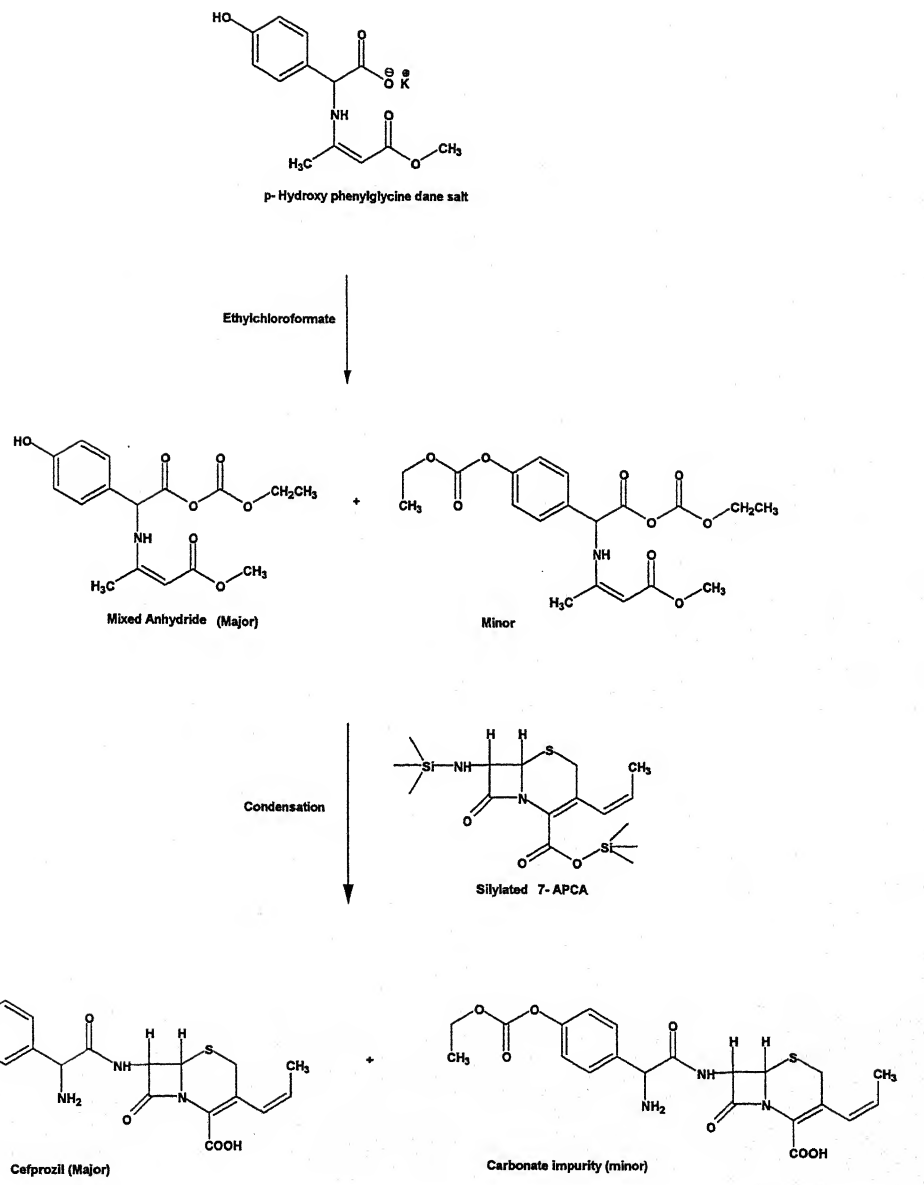
CONCLUSION

Based on analysis of the consolidated spectral information, the structure of the impurity at RRT~ 1.59 was proposed as depicted below Cefprozil carbonate,



(6R)-3-((1Z)prop-1-enyl)-6-[2-amino-2-(4-ethoxycarbonyloxyphenyl) acetylamino]-5-oxo-2H,6H,6aH-azetidino[2,1-b]1,3-thiazine-4-carboxylic acid.(Cefprozil Carbonate)

PROPOSED FORMATION PATHWAY OF IMPURITY



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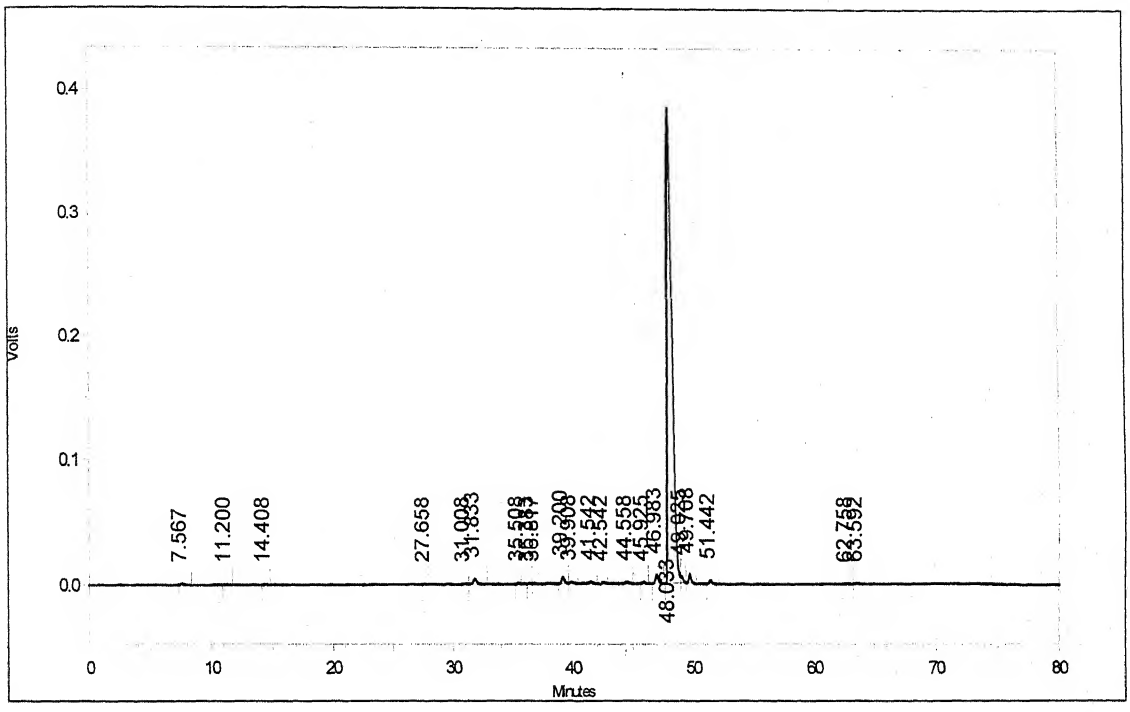
Aquired Time: 3/19/2004 1:50:25 PM
Sample ID : Cefprozil Carbonate



Detector A (280nm)

Pk #	Retention Time	Area	Area Percent	Name
1	7.567	17265	0.15	
2	11.200	6423	0.06	
3	14.408	5163	0.05	
4	27.658	5073	0.04	
5	31.008	6006	0.05	
6	31.833	92792	0.82	
7	35.508	8456	0.07	
8	36.383	4727	0.04	
9	36.817	5845	0.05	
10	39.200	80255	0.71	
11	39.908	23255	0.20	
12	41.542	24302	0.21	
13	42.542	12328	0.11	
14	44.558	20138	0.18	
15	45.925	13328	0.12	
16	46.983	113452	1.00	
17	48.033	10684921	94.06	Cefprozil Carbonate
18	49.025	65694	0.58	
19	49.708	106431	0.94	

Method Name : C:\HPLC-23\Method\Cefrozil\Cefprozil RS.met
File Name: C:\HPLC-23\Data\March.04\Cefprozil\031904.03
Aquired Time: 3/19/2004 1:50:25 PM
Sample ID : Cefprozil Carbonate

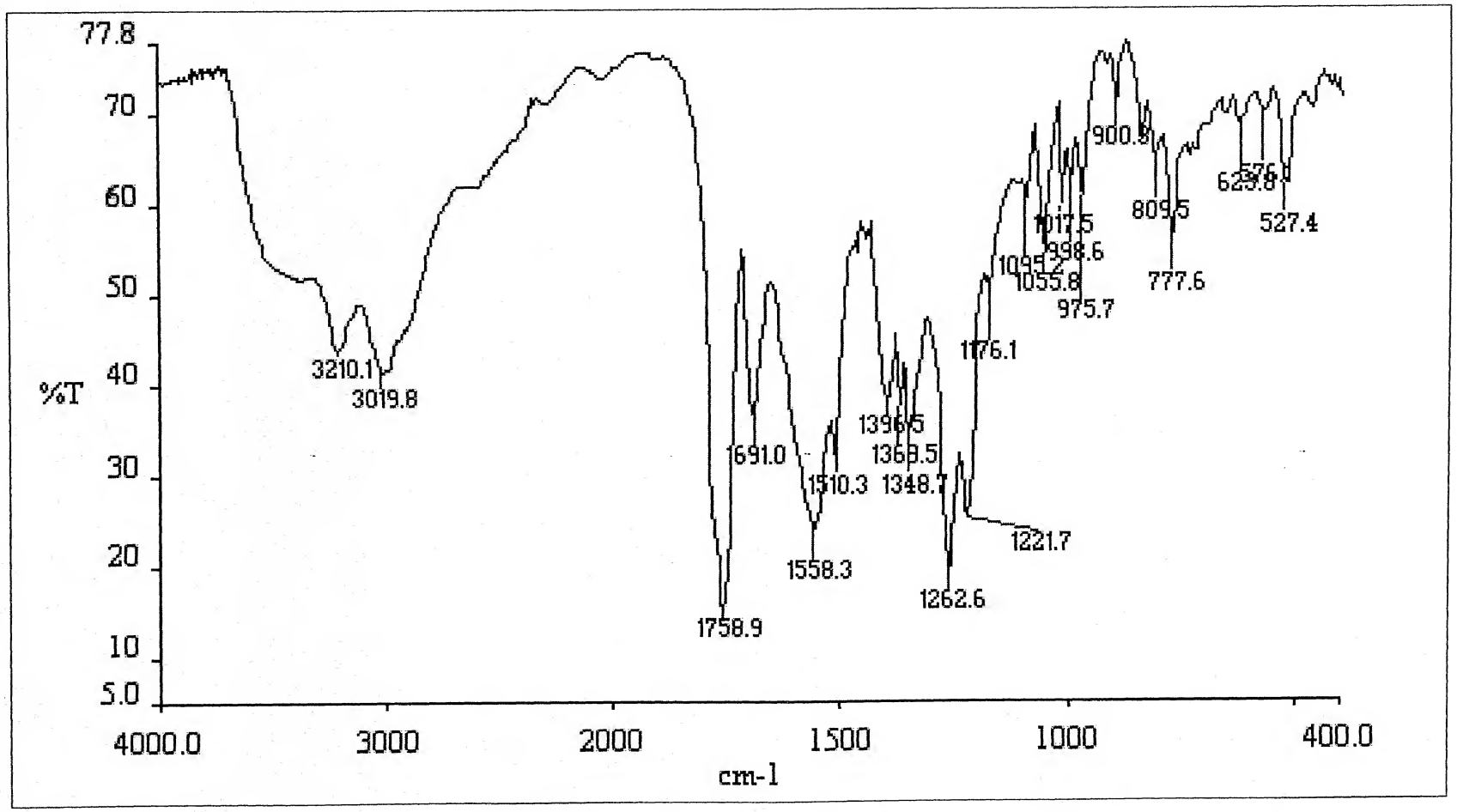
20	51.442	45369	0.40
21	62.758	8398	0.07
22	63.592	10550	0.09

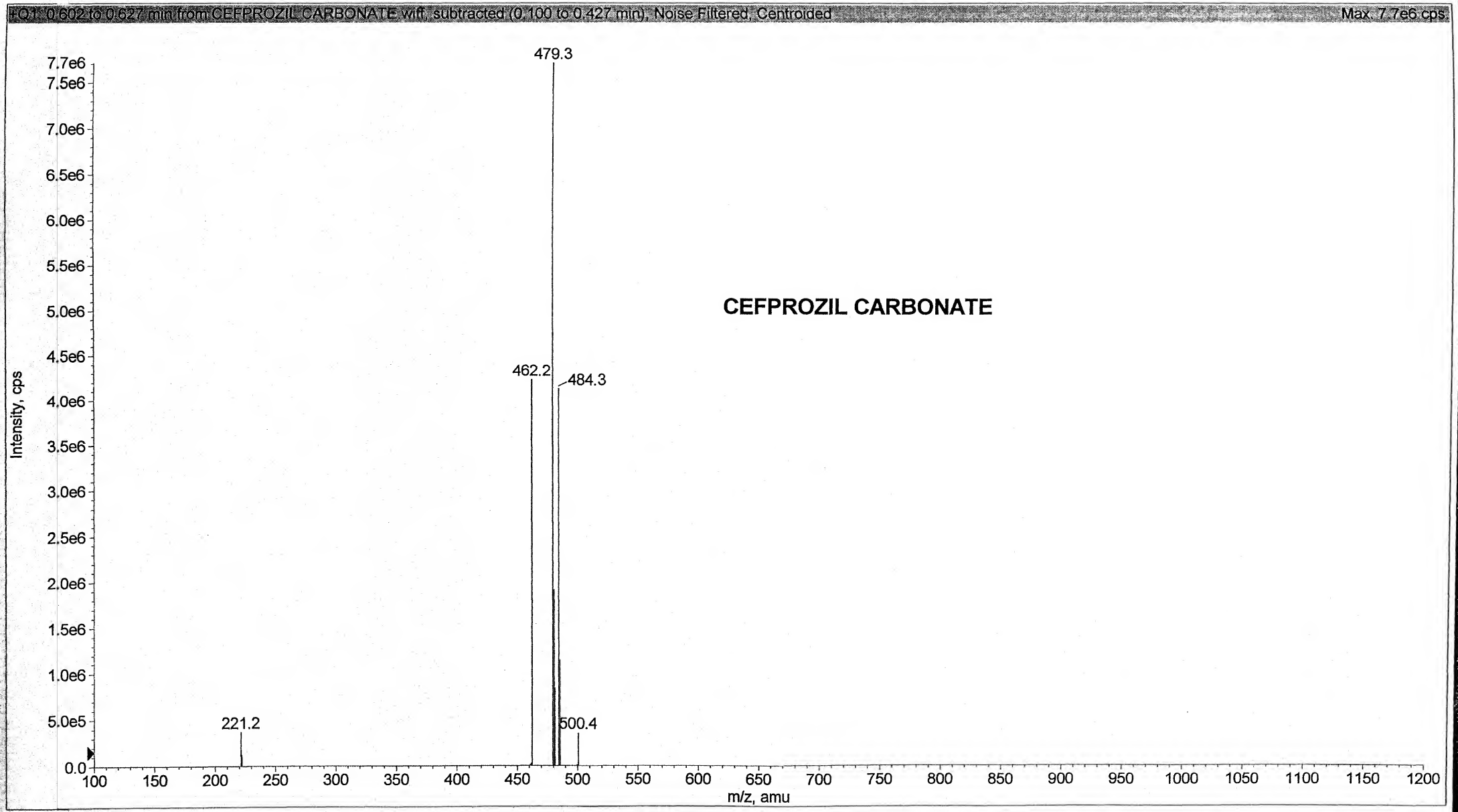
Totals		11360171	100.00	

Cefprozil Carbonate in DMSO-d6
8.883
8.453
7.502
7.416
7.373
7.257
7.214
6.395
6.338
5.754
5.716
5.419
5.384
5.362
5.327
5.110
4.555
4.517
4.470
4.304
4.268
4.233
4.198
3.632
3.545
3.516
3.342
2.500
1.661
1.654
1.625
1.326
1.291
1.255
ppm
0.611
0.593
0.329
1.461
2.189
0.568
0.696
0.833
0.804
1.378
2.127
2.076
3.000
2.455

INSTRUMENT : PERKIN ELMER 1600 Series FTIR

Sample ID : Cefprozil Carbonate





Sample Name: CEFPROZIL CARBONATE

OBJECTIVE: - IDENTIFICATION OF IMPURITY AT RRT- 0.73

To identify the Impurity present at RRT ~ 0.73 spectroscopic (LC-MS, LC-MS-MS, PDA) analysis of the sample RG/009/NDM/035/031 is performed.

LC-MS:

An in-house LC-MS method was developed to analyze the impurity at RRT ~ 0.72. Following conditions were used,

Chromatographic conditions:

Column

- Type: Inertsil ODS 3V
- Dimensions: 250 mm x 4.6 mm
- Particle size: 5µm

Detector setting

- Wavelength: 280 nm

Mobile Phase

- Buffer: 0.01 M Ammonium Acetate
- Mobile A: Buffer: ACN (98:02 v/v)
- Mobile B: Buffer: ACN (30:70 v/v)
- pH: As such
- Flow rate: 1.5 ml/min

The solutions were filtered and each mobile phase was de-aerated separately,

MS Conditions:

DP	FP	IVE	TEMP.	NEB	CUR
6	60	3500	250	13	7

Chromatographic condition

S.No.	Time	Event	Value	Flow ml/min
1.	0.01	B.conc	0.00	1.5
2.	15.00	B.conc	5.00	1.5
3.	35.00	B.conc	25.00	1.5
4.	70.00	B.conc	50.00	1.5
5.	72.00	B.conc	0.00	1.5
6.	80.00	B.conc	0.00	1.5

LC-MS analysis of the sample exhibited a peak at RT 15.43 min. of 364.1 m/z, amu, therefore, the molecular weight of the impurity is 363, could be of Cefadroxil. The sample RG/009/NDM/035/031 was subjected to LC-MS-MS analysis to check the fragmentation pattern of the peak at m/z 364.1, which showed fragments of m/z 347.2, 208.1, 190.2, 157.9, 140.3 and 114 respectively.

LC-MS-MS analysis of the standard Cefadroxil was also carried out simultaneously to compare the fragmentation pattern with that of impurity at RT 19.98 min. It is observed that the standard Cefadroxil molecule exhibits a molecular ion peak at m/z, 364.1 and fragment peaks at m/z 347.2, 208.1, 190.2, 158.1, 140.0 and 114.2. The fragmentation pattern of the standard Cefadroxil molecule is exactly matching with the peak appeared for the impurity of m/z 364.1.

Photo Diode Array (PDA) Analysis

An in-house HPLC method was developed to analyze the sample RG/009/NDM/035/031 for PDA matching of the peak eluting at RRT ~ 0.73 with standard Cefadroxil. Following chromatographic condition was used.

Chromatographic condition:

Column

- Type: Inertsil C_{18}
- Dimensions: 250mm x 4.6 mm
- Particle size: 5µm

Detector setting

- Wavelength: 190-370 nm

Mobile phase

- Mobile A: Dissolve 1 ml of orthophosphoric acid (85%) in 1000 ml HPLC grade water.
- Mobile B: Methanol HPLC grade
- Flow rate: 1.5 ml/min

The solutions were filtered and each mobile phase was de-aerated separately.

Chromatographic condition

S.No.	Time	Event	Value	Flow ml/min
1.	0.01	B.conc	7	1.5
2.	3.00	B.conc	7	1.5
3.	12.00	B.conc	7	1.5
4.	70.00	B.conc	75	1.5
5.	72.00	B.conc	7	1.5
6.	80.00	B.conc	7	1.5
7.	80.01	stop		

PDA analysis showed a similarity Index value of 0.999 for the peak eluting at RRT ~ 0.73 to that of standard Cefadroxil. This further confirms that the Impurity present in the sample is Cefadroxil.

SPECTRAL ANALYSIS

NMR SPECTRA

The NMR spectra of impurity at RRT ~ 0.73 was recorded on a 200 MHz Bruker DRX-200 instrument using DCl.

^{1}H NMR

7.34-7.15 (d, 2H); 6.79-6.75 (d, 2H); 5.44-5.42(d, 1H); 5.09 (s, 1H); 4.91 (d, 1H); 3.31-3.0 (q, 2H); 1.89 (s, 3H) ppm/δ

IR in KBr

The IR spectrum was recorded on FTIR-8201PC Shimadzu instrument using KBr pallet.

3492.8; 3205.7; 2908.6; 1759.7; 1682.3; cm^{-1}

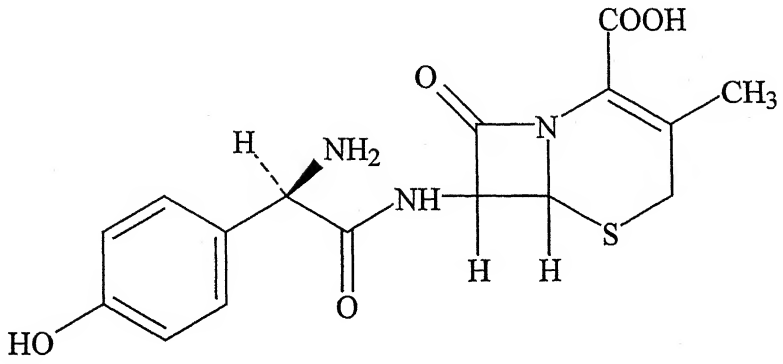
MASS SPECTRA

The compound exhibited a molecular ion peak at 364.1, implying a molecular weight 363. The mass spectrum was recorded on PE SCIEX API-3000 triple quadrupole mass spectrometer. For mass spectrum the sample was dissolved in methanol and spectrum was taken using Atmospheric Pressure Ionization technique having turbolon ionization source. The declustering potential was set at 6v; focusing potential at 60v, and ionization volt energy (IVE) 4000 at 350°C.

Molecular weight	Assignment
364	$(M+H)^+$ Molecular ion

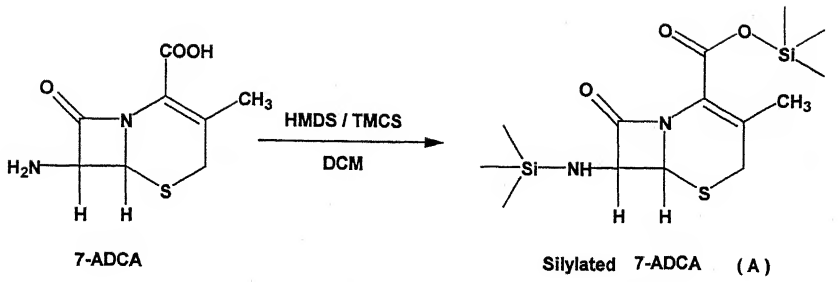
CONCLUSION

Based on analysis of the consolidated spectral information, the structure of the impurity at RRT~ 0.73 was proposed as depicted below,



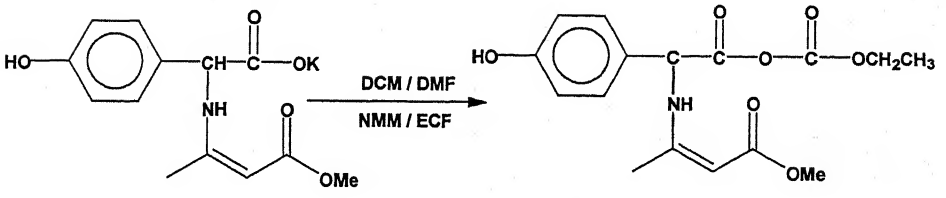
(6R, 7R)-7-[[(2R)-2-Amino-2- (4-hydroxyphenyl) acetyl] amino]-3-methyl-8-oxo-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid. (Cefadroxil)

SYNTHETIC PATHWAY OF IMPURITY



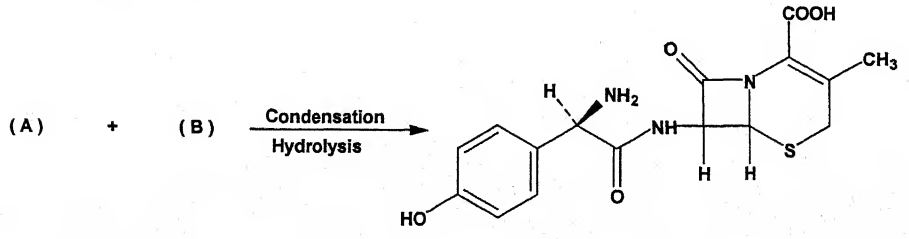
7-ADCA

Silylated 7-ADCA (A)

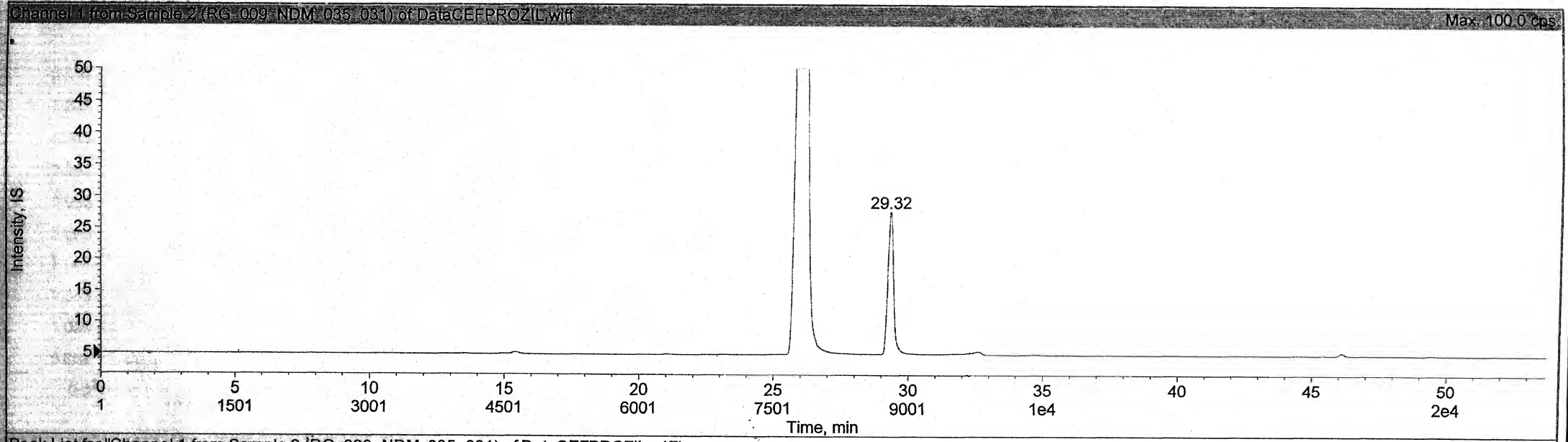


K Dane Salt

Mixed Anhydride (B)

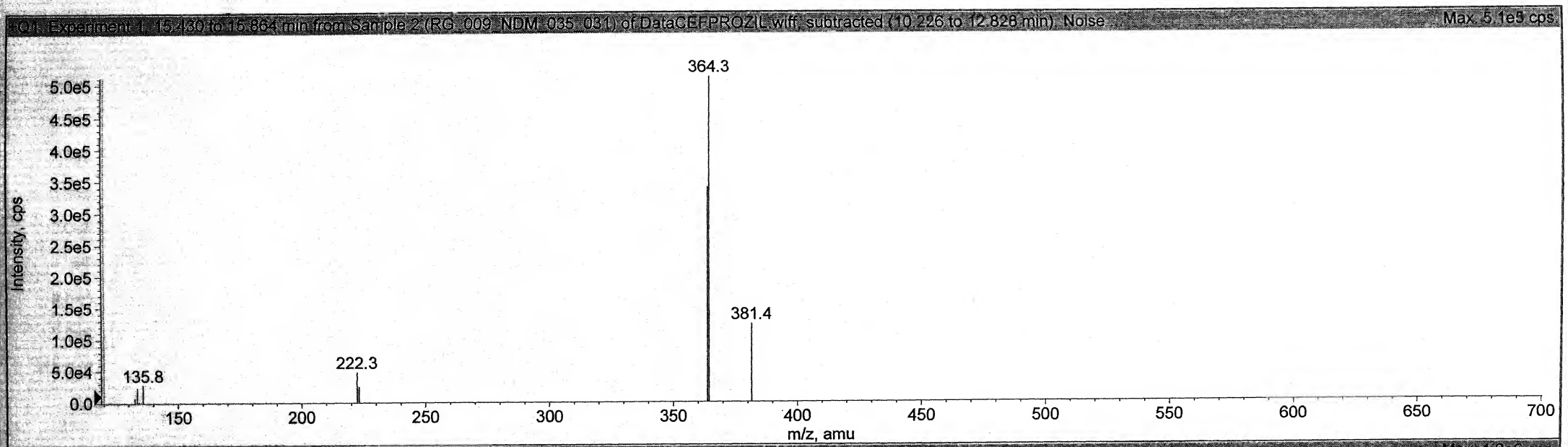


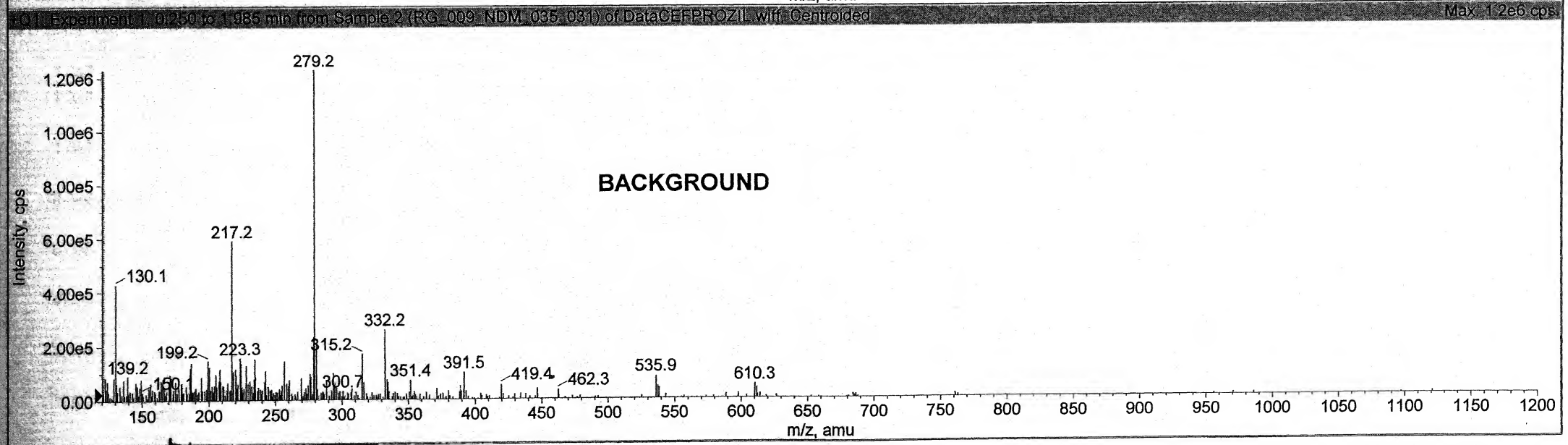
Cefadroxil



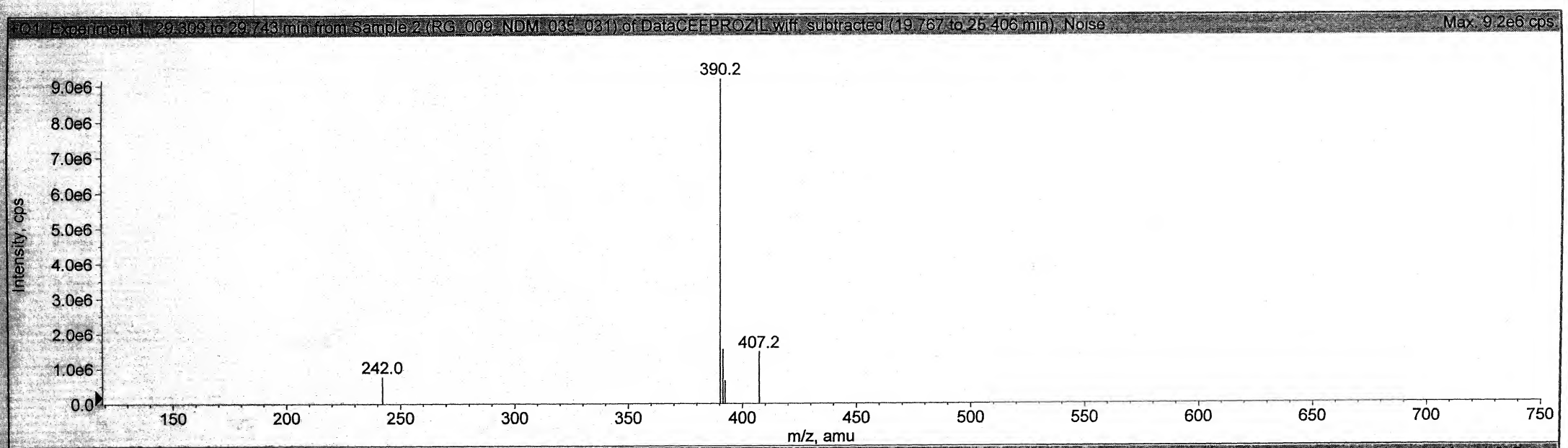
Peak List for "Channel 1 from Sample 2 (RG_009_NDM_035_031) of DataCEFPROZIL.wiff"

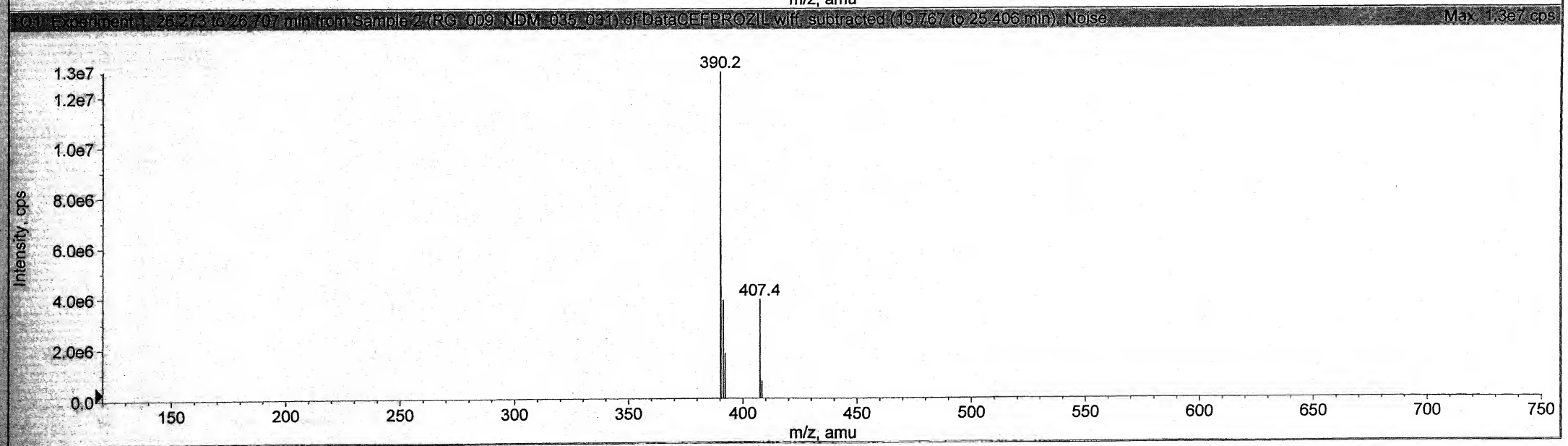
	Time (min)	Area (counts)	% Area	Height (cps)	% Height	Width (min)	Baseline Type
1	14.96	2.45	0.08	0.16	0.13	0.49	Valley
2	15.44	7.45	0.24	0.36	0.30	0.81	Valley
3	21.08	3.88	0.12	0.21	0.17	0.68	Base to Base
4	25.88	2743.39	88.04	95.14	79.57	2.89	Base to Base
5	29.32	341.13	10.95	22.69	18.98	1.38	Base to Base
6	32.57	9.71	0.31	0.49	0.41	0.68	Base to Base
7	46.11	8.13	0.26	0.52	0.43	0.70	Base to Base

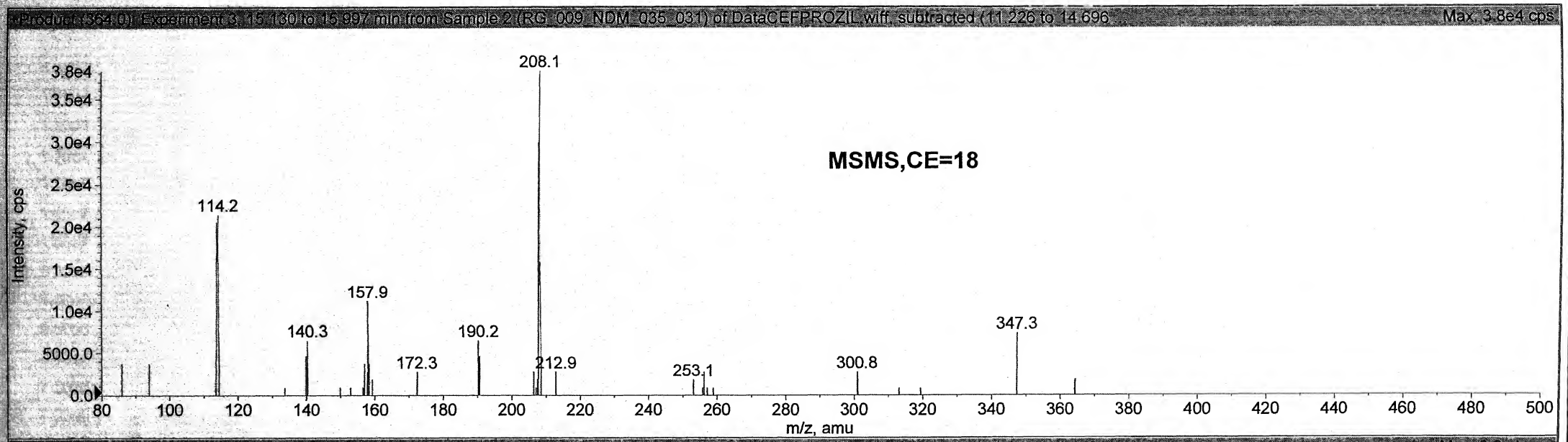

+Q1 Experiment 1, 15.430 to 15.864 min from Sample 2 (RG_009_NDM_035_031) of DataCEFPROZIL.wiff, subtracted (10.226 to 12.828 min), Noise
Max. 5.1e5 cps.
Intensity, cps
m/z, amu
364.3
381.4
222.3
135.8

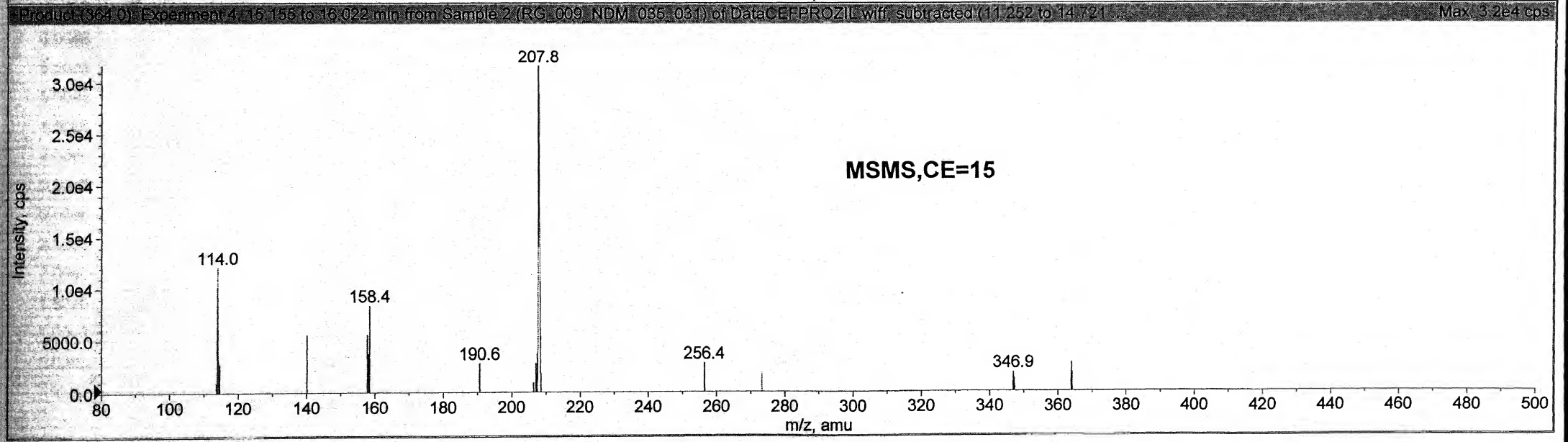

+Q1 Experiment 1, 0.250 to 1.985 min from Sample 2 (RG_009_NDM_035_031) of DataCEFPROZIL.wiff, Centroided
Max. 1.2e6 cps.
Intensity, cps
m/z, amu
BACKGROUND
279.2
217.2
130.1
332.2
315.2
199.2
223.3
139.2
160.1
300.7
351.4
391.5
419.4
462.3
535.9
610.3

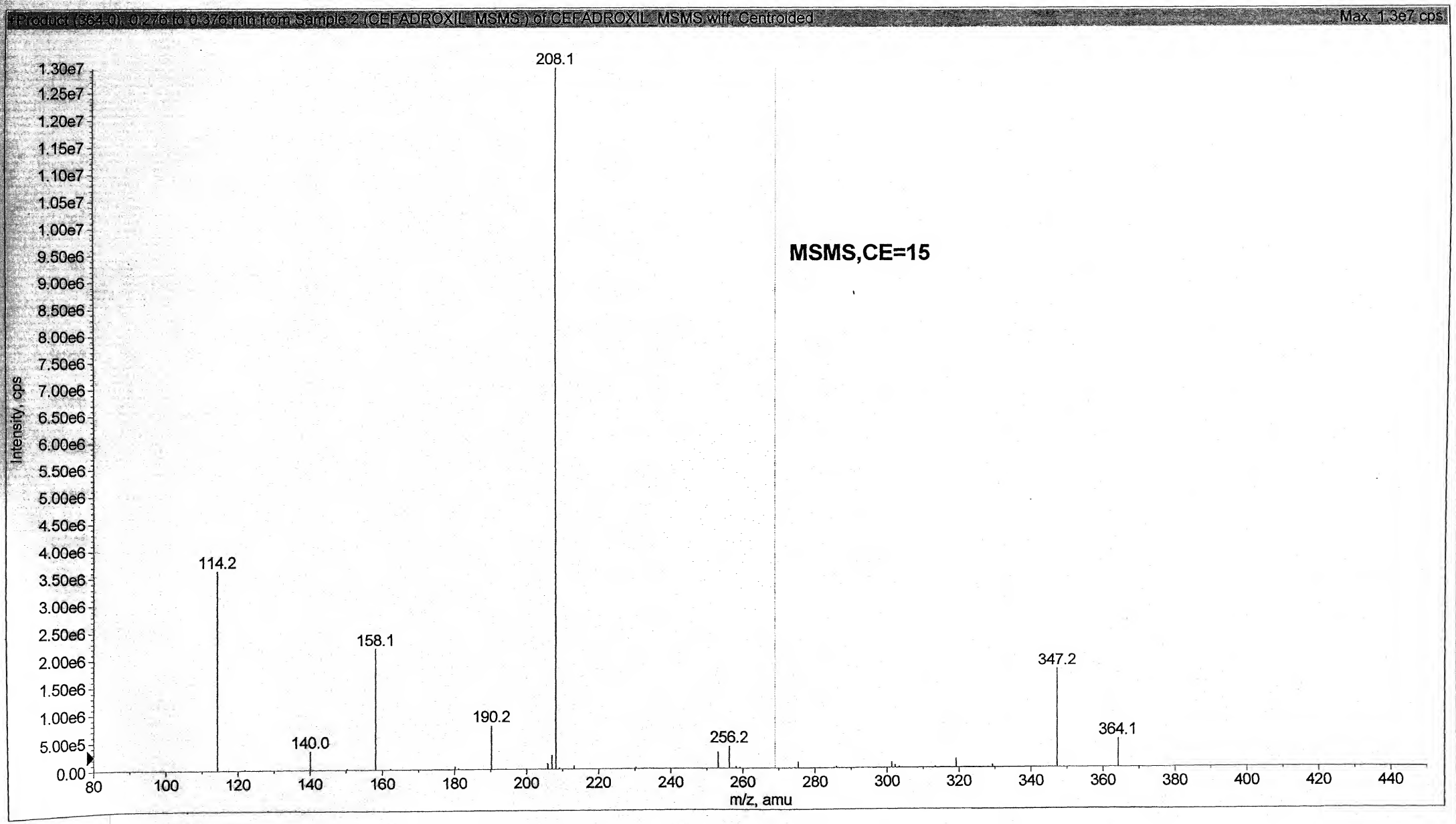
183

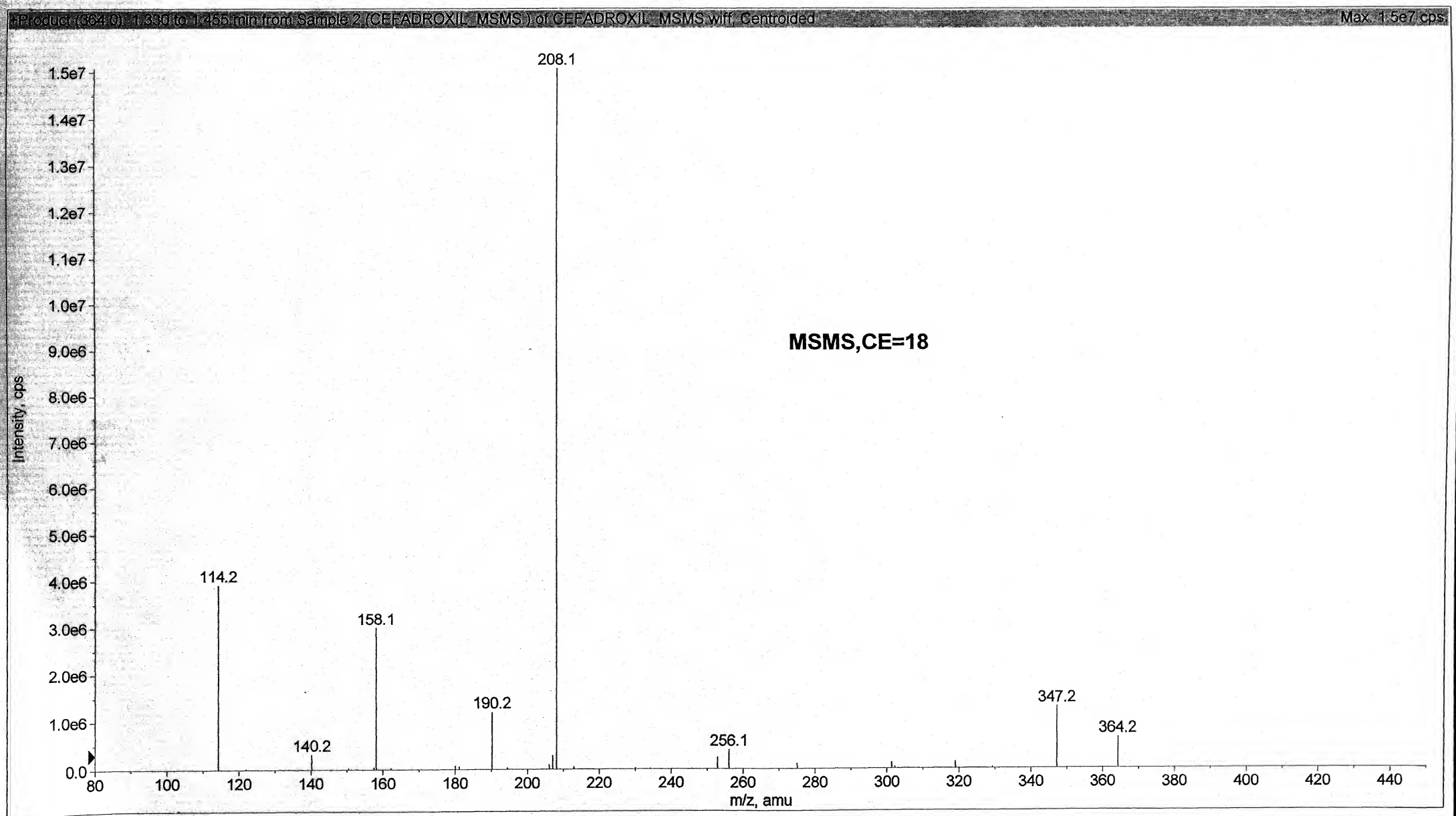

+Q1 Experiment 1, 29.309 to 29.743 min from Sample 2 (RG_009_NDM_035_031).wiff, subtracted (19.767 to 25.406 min), Noise ...
Max. 9.2e6 cps.
390.2
407.2
242.0
Intensity, cps
m/z, amu


+Q1 Experiment 1, 26.273 to 26.707 min from Sample 2 (RG_009_NDM_035_031).wiff, subtracted (19.767 to 25.406 min), Noise ...
Max. 1.3e7 cps.
390.2
407.4
Intensity, cps
m/z, amu


Product (364.0): Experiment 3, 15.130 to 15.997 min from Sample 2 (RG_009_NDM_035_031) of DataCEFPROZIL.wiff, subtracted (11.226 to 14.696
Max. 3.8e4 cps
MSMS,CE=18
Intensity, cps
m/z, amu
208.1
114.2
157.9
140.3
190.2
172.3
212.9
253.1
300.8
347.3


Product (364.0): Experiment 4, 15.155 to 16.022 min from Sample 2 (RG_009_NDM_035_031) of DataCEFPROZIL.wiff, subtracted (11.252 to 14.721
Max. 3.2e4 cps
MSMS,CE=15
Intensity, cps
m/z, amu
207.8
114.0
158.4
190.6
256.4
346.9

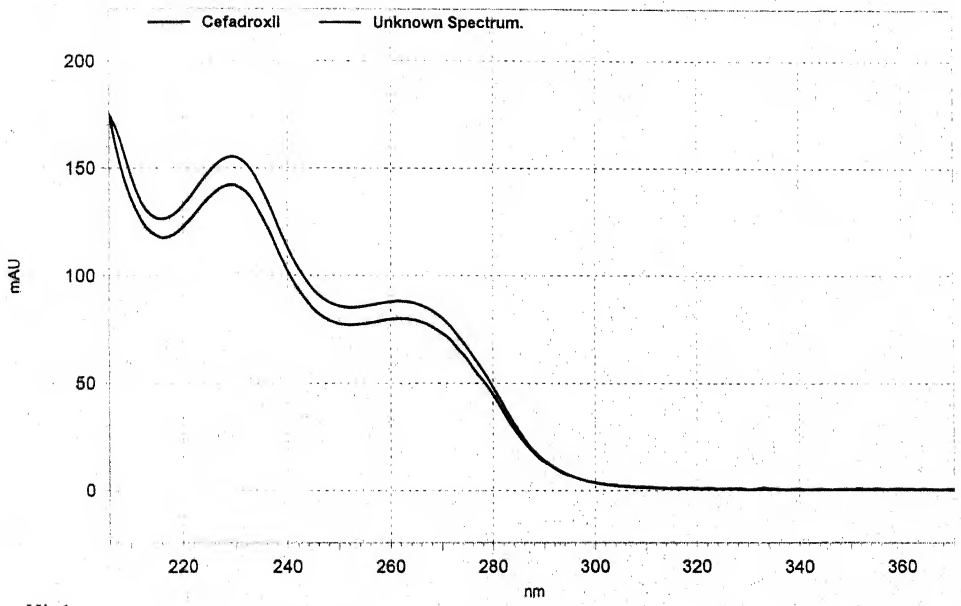

Product (564.0), 0.276 to 0.376 min from Sample 2 (CEFADROXIL_MSMS_) of CEFADROXIL_MSMS.wiff, Centroided
Max. 1.3e7 cps.
MSMS,CE=15
Intensity, cps
m/z, amu
208.1
114.2
158.1
190.2
140.0
256.2
347.2
364.1


+Product (364.0) 1.330 to 1.455 min from Sample 2 (CEFADROXIL_MSMS) of CEFADROXIL_MSMS.wiff, Centroided
Max. 1.5e7 cps.
MSMS,CE=18
Intensity, cps
m/z, amu
208.1
114.2
158.1
190.2
140.2
256.1
347.2
364.2

187

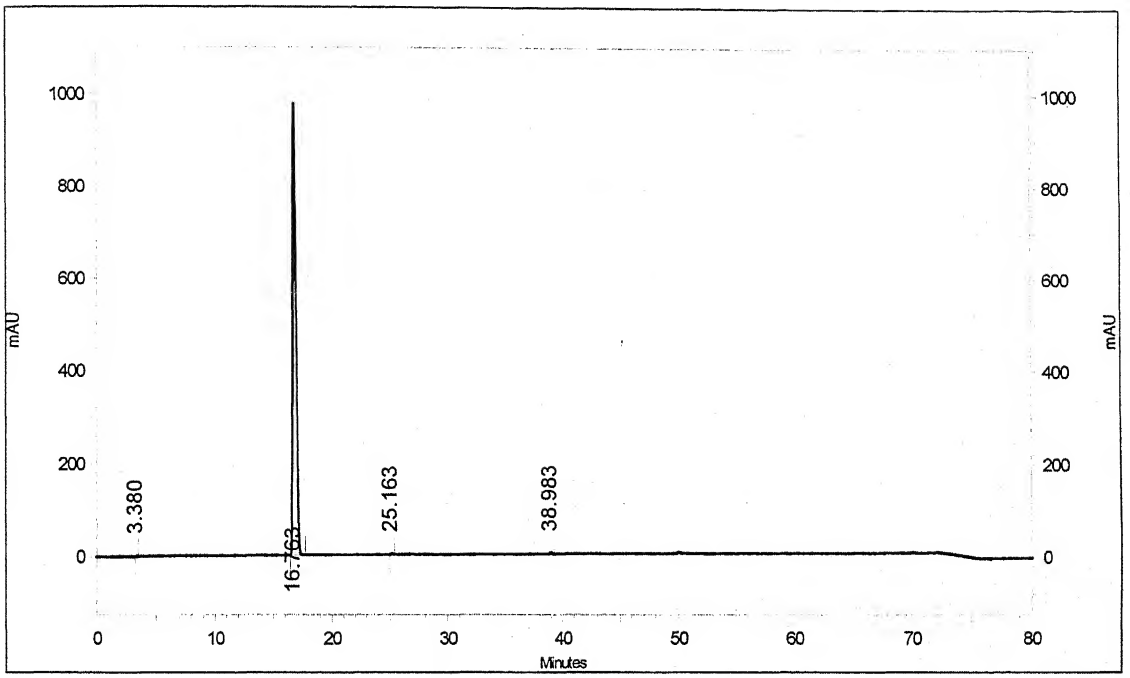
Method Name:	**D:\CLASSvp\PDA-LC33\Method\Cefprozil\046 (GRD)2.met**
File Name:	**D:\CLASSvp\PDA-LC33\Data\Feb-04\Cefprozil\022604.16**
Aquired Time:	**2/27/2004 10:51:21 AM**
Sample ID:	**Cefprozil MH (5000 ppm)**

Retention Time: 19.48 min. **Number of Hits: 1**



Hit 1
Name: Cefadroxil
Similarity Index: 0.999655
Library: D:\CLASSvp\PDA-LC33\Library\Cefprozil\Individual imp\Individual imp..lib

Method Name: E:\HPLC-24\Method\Cefprozil\Cefprozil.met
File Name: E:\HPLC-24\Data\March 04\Cefprozil\030904.6
Aquired Time: 3/10/2004 12:21:45 AM
Sample ID: Cefadroxil



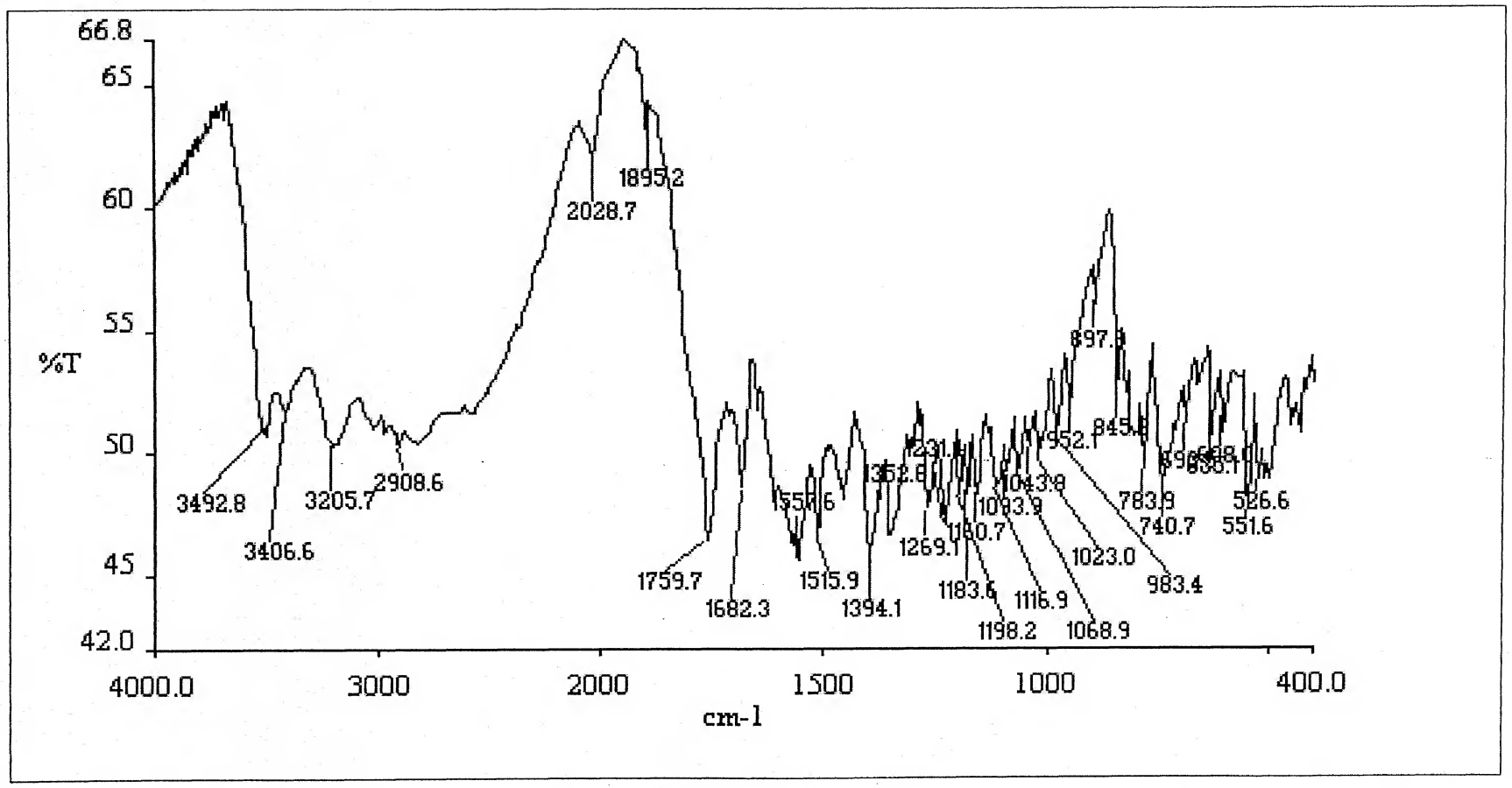
Detector 1-280nm
Results
(Reprocessed)

Pk #	Retention Time	Area	Area Percent	Name
1	3.380	9580	0.05	
2	16.763	19273159	99.75	**Cefadroxil**
3	25.163	15769	0.08	
4	38.983	23262	0.12	
Totals		19321770	100.00	

Cefadroxil in DCL
7.349
7.250
7.188
7.150
6.794
6.751
5.446
5.424
5.094
4.911
4.889
3.313
3.222
3.092
3.001
1.890
2.869
1.021
0.929
0.980
0.947
0.975
3.389
2.082
ppm
9
8
7
6
5
4
3
2

INSTRUMENT : PERKIN ELMER 1600 Series FTIR

Sample ID : Cefadroxil



OBJECTIVE: - ISOLATION OF IMPURITY AT RRT~ 1.17

This impurity at RRT ~ 1.17 was isolated from cefprozil sample using preparative HPLC.

Preparative isolation

The chromatographic condition used was as follows:

Column

- Type: X-Terra C_{18}
- Dimensions: 300 mm x 19 mm
- Particle size: 7 μm

Detector setting

- Wavelength: 280 nm
- Flow rate: 15 ml/min

Mobile Phase

- Mobile A: Water (0.2% acetic Acid)
- Mobile B: Acetonitrile

Sample preparation

~130 mg of sample dissolved in 8 ml-distilled water+ 10% ammonia 2 drops.

Loading amount: 130 mg sample/injection

Chromatographic condition

S.No.	Time	Event	Value	Flow ml/min
1.	0.01	B.conc	0.00	15.0
2.	5.00	B.conc	0.00	15.0
3.	10.00	B.conc	15.00	15.0
4.	15.00	B.conc	20.00	15.0
5.	20.00	B.conc	25.00	15.0
6.	25.00	B.conc	35.00	15.0
7.	35.00	B.conc	70.00	15.0
8.	37.00	B.conc	100.00	15.0
9.	39.00	B.conc	0.00	15.0
10.	55.00	B.conc	Stop	

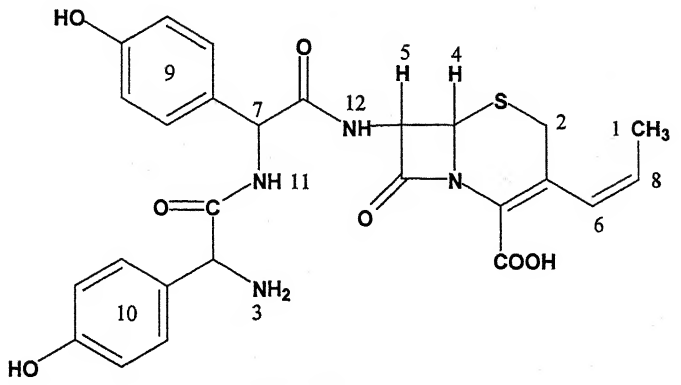
Fractions collected were monitored using the analytical method. Fractions having the impurity sample in (~97%) purity were concentrated using rotary evaporator at bath temperature 35°C under vacuum to distill out acetonitrile. The combined fractions were then lyophilized to furnish ≥97 % (By HPLC area normalization method) pure material, as white solid.

The solid obtained was taken for further evaluation of the purity and also for various spectroscopic studies.

SPECTRAL ANALYSIS

NMR SPECTRA

The NMR spectra of impurity at RRT ~ 1.17 was recorded on a 200 MHz Bruker DRX-200 instrument using DMSO-d6.



^{1}H NMR in DMSO-d6

δ (ppm)	Relative protons	Proton Assignment
8.96-8.85	2	12,11
7.26	4	10, (Phenyl protons)
6.77	4	9,(Phenyl protons)
6.31	1	8
5.55-5.34	3	7,6,5
4.94	1	4
4.71	2	3
3.59-3.28	2	2
1.66	3	1

IR in KBr

The IR spectrum was recorded on FTIR-8201PC Shimadzu instrument using KBr pallet.

Frequency cm^{-1}	Assignment
3380.8	-OH stretch
3242.3	-N-H stretch
1769.2	β-Lactam C=O
1651.2	CONH

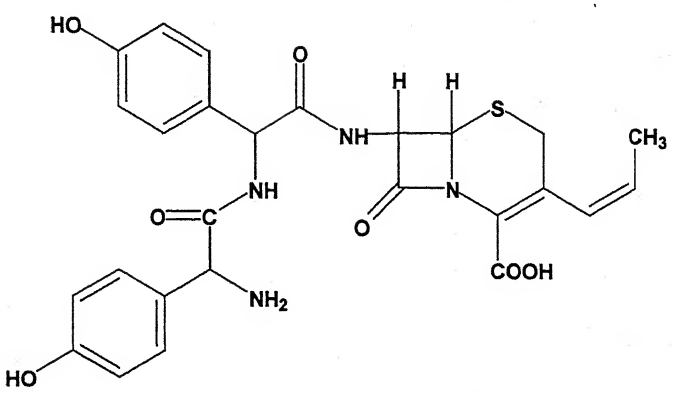
Mass m/z amu

The compound exhibited a quasi-molecular ion peak at 539 implying a molecular weight of 538. The mass spectrum was recorded on PE SCIEX API-3000 triple quadruple mass spectrometer. For mass spectrum the sample was dissolved in methanol and spectrum was taken using Atmospheric Pressure Ionization technique having turbolon ionization source. The declustrring potential was set at 6 v; focusing potential at 60v, and ionization volt energy (IVE) 4000 at 400°C

Molecular weight	Assignment
539.4	$(M+H)^+$
561.3	(M+Na)

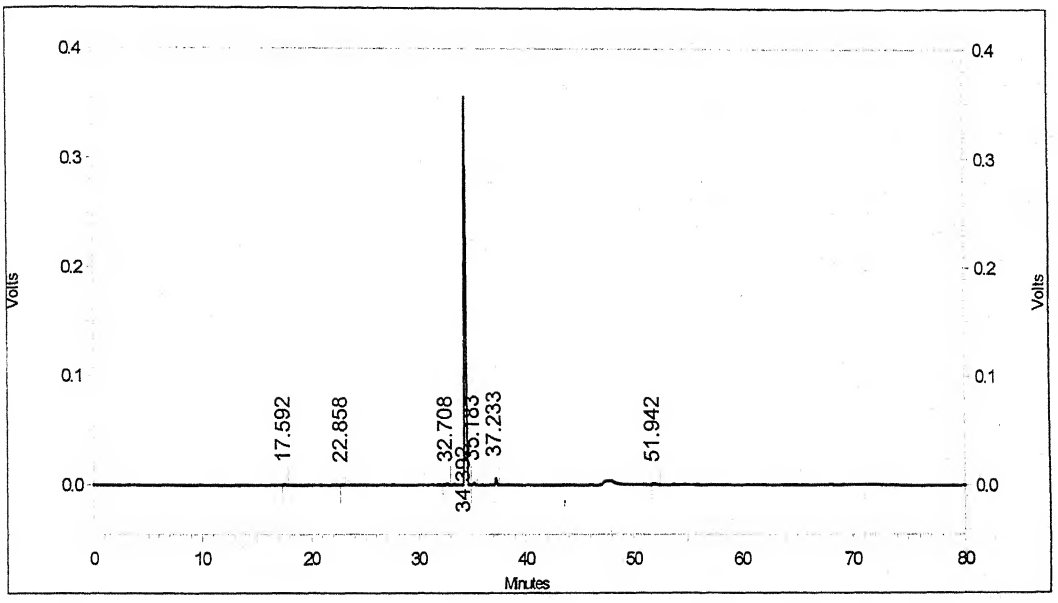
CONCLUSION

Based on analysis of the consolidated spectral information, the structure of the impurity at RRT~ 1.17 was proposed as depicted below,



3-((1Z)prop-1-enyl)-6-{2-[2-amino-2-(4-hydroxyphenyl)acetylamino]-2-(4-hydroxyphenyl)acetylamino}-5-oxo-2H,6H,6aH-azetidino[2,1-b]1,3-thiazine-4-carboxylic acid. (PHPG Cefprozil)

Method Name: D:\CLASSvp\UV-LC33\Method\Cefprozil\Cefprozil.met
File Name: D:\CLASSvp\UV-LC33\Data\May-04\Cefprozil\050504.03
Aquired Time: 5/5/2004 2:41:43 PM
Sample ID: PHPG Cefprozil (Z – Isomer)

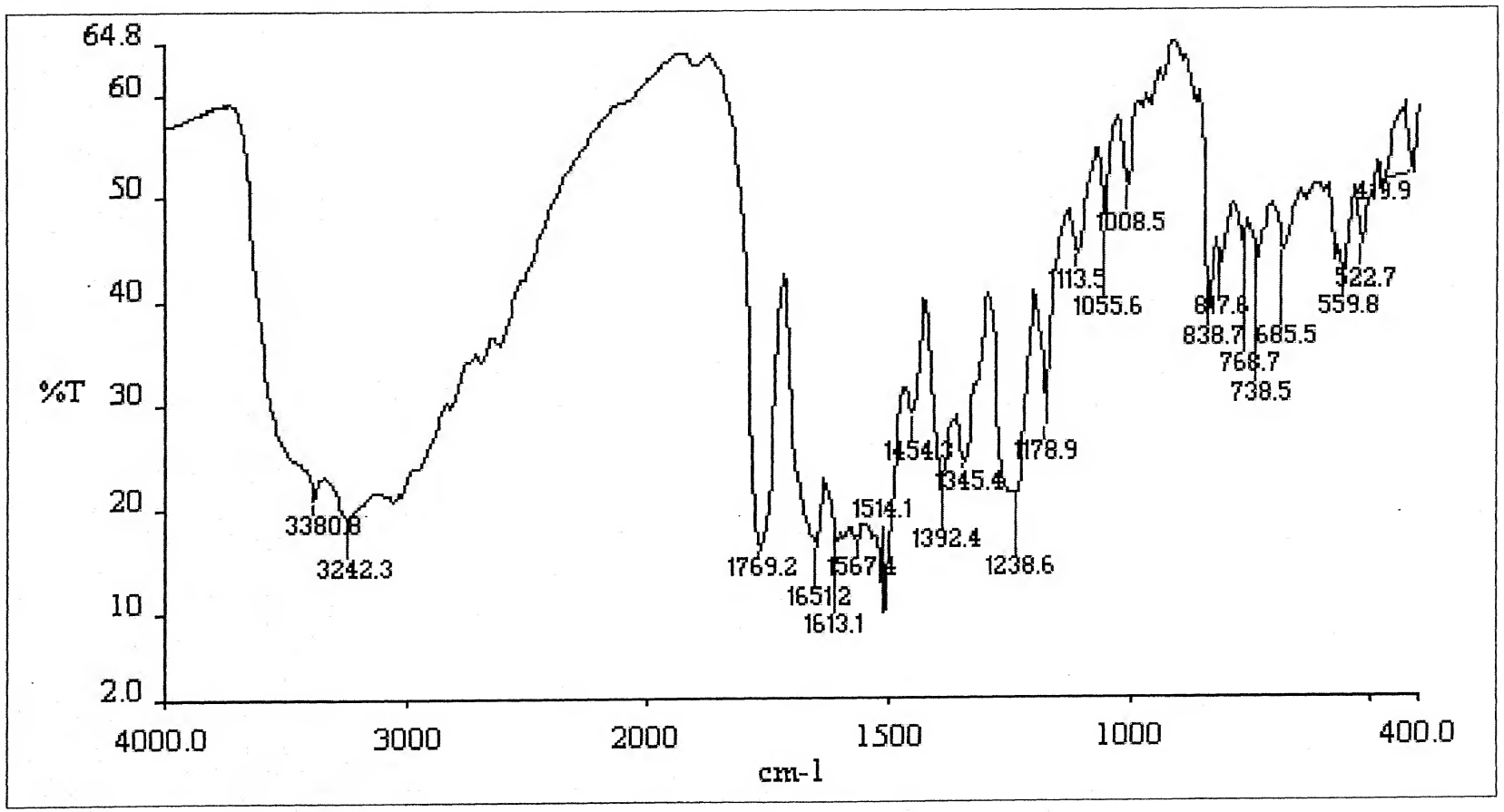


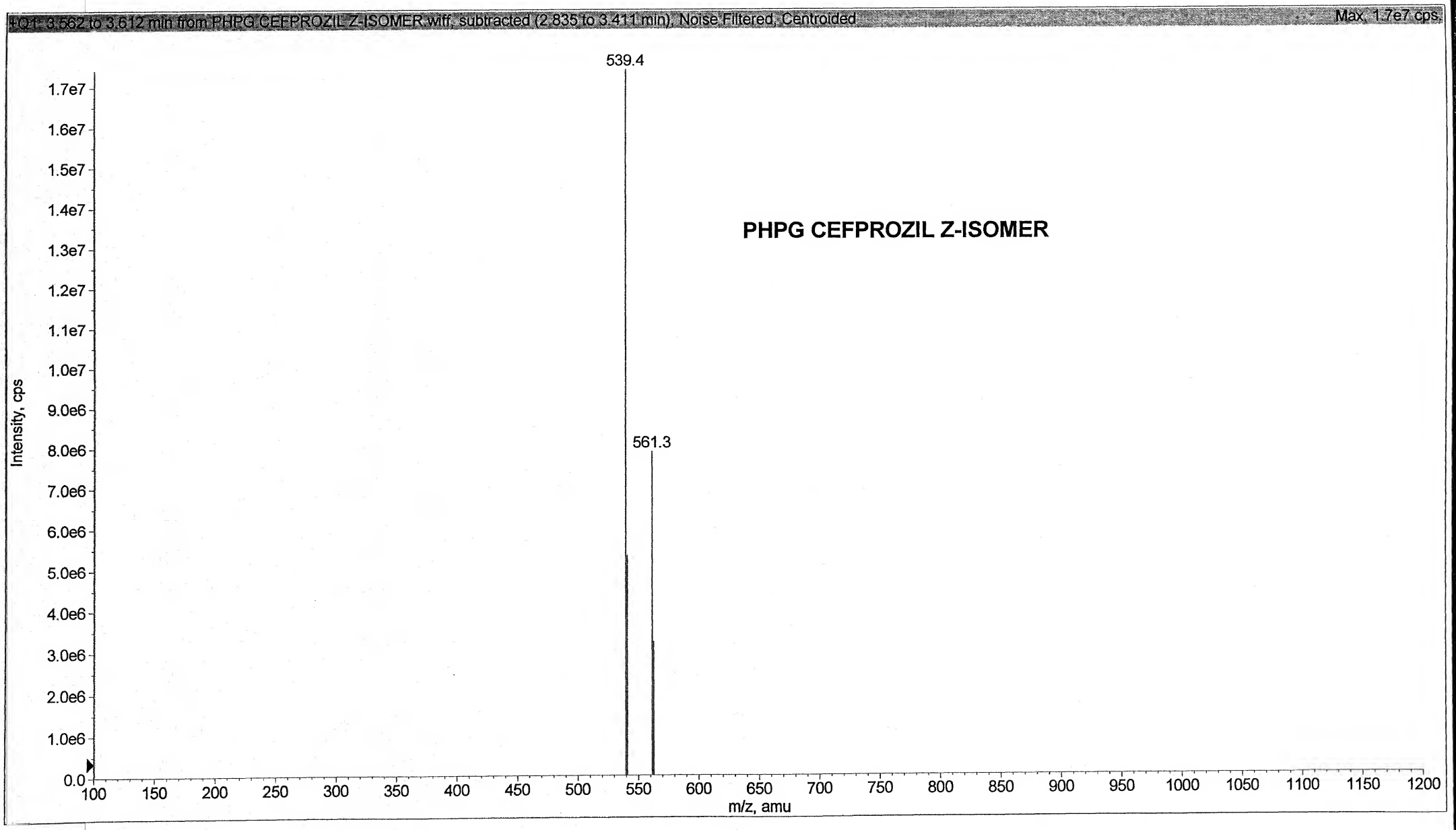
Detector B (280nm) Pk #	Retention Time	Area	Area %	Name
1	17.592	7674	0.19	
2	22.858	5440	0.13	
3	32.708	13637	0.33	
4	34.392	3968560	97.23	**PHPG Cefprozil (Z – Isomer)**
5	35.183	17396	0.43	
6	37.233	58172	1.43	
7	51.942	10810	0.26	
Totals		4081689	100.00	

PHPG Cefprozil(Z-Isomer) in DMSO-d6
8.938
8.896
8.831
7.229
7.188
7.148
6.734
6.687
6.643
6.280
6.221
5.515
5.481
5.434
5.399
5.376
5.340
5.304
4.902
4.879
4.688
3.561
3.475
3.328
3.243
2.462
2.033
1.867
1.627
1.592
1.000
2.031
1.093
0.521
1.664
1.227
1.819
1.350
0.238
1.331
ppm

INSTRUMENT : PERKIN ELMER 1600 Series FTIR

Sample ID : PHPG Cefprozil (Z – Isomer)





Sample Name: PHPG CEFPROZIL Z-ISOMER

SUMMARY OF THE RESEARCH WORK

This research work was aimed to isolate different impurities, which are present 0.1% in amount both at initial stage as well as after accelerated storage conditions. The products chosen for the studies are ceftiofur, cefuroxime axetil, and cefprozil. Suitable preparative HPLC methods are developed for the isolation of impurities and their structures have been assigned using various instrumentation techniques.

Impurities

Impurities in pharmaceuticals are the unwanted chemicals that remain with the active pharmaceutical ingredients (APIs). These unwanted chemicals can be process impurities, or developed during formulation or upon aging of both API and drug products. The presence of these unwanted chemicals even in small amounts may influence the efficacy and safety of the pharmaceutical products. There is a good significant demand for the impurity reference standards along with and the API reference standards for both regulatory authorities and pharmaceutical companies. According to International Conference of Harmonization (ICH) guidelines on impurities in new drug products, identification of impurities below than 0.1% level is not considered to be necessary

unless the potential impurities are expected to be unusually potent or toxic. In all cases, impurities should be qualified. If data are not available to qualify the proposed specification level of an impurity studies to obtain such data may be needed (when the usual qualification threshold limits given below are exceeded). According to ICH, the maximum daily dose qualification threshold is considered as follows:

≤ 2g/day 0. 1 % or 1 mg per day intake (whichever is lower)

≥ 2g/day 0.05%

Sources of Impurities in Medicines

Medicines are the formulated forms of active pharmaceutical ingredients. There are two types of impurities in medicines:

1. Impurities associated in with active pharmaceutical ingredients
2. Impurities that form are created during formulation and / or with aging or that are related to the formulated forms.

1. Impurities associated in with APIs

According to ICH guidelines, impurities associated with APIs are classified into the following categories:

- Organic impurities (Process related)
- Inorganic impurities
- Residual solvents

Organic impurities

Organic impurities may arise during the manufacturing process, or degradants. They may be known or unknown, volatile or non-volatile and include the following:

- Starting materials or intermediates
- By-products
- Degradation products
- Reagents, ligands, and catalysts
- Enantiomeric impurities

Inorganic impurities

Inorganic impurities may also be derived from the manufacturing processes used for bulk drugs. Normally they are related to:

- Reagents, ligands and catalysts
- Heavy metals
- Other materials (e.g., filter aids, charcoal)

Residual solvents

Residual solvents are organic volatile chemicals used during the manufacturing process or generated during the production. It is very difficult to remove these solvents completely by the work-up process or by the drying of API, however; efforts should be taken to the extent possible to meet the safety data requirements. Residual solvents are divided into three classes. Solvents such as benzene (Class I, 2 ppm limit) and carbon tetrachloride (Class I, 4 ppm limit) are to be avoided. On the other hand, the limit of most commonly used solvents are methylene chloride (600 ppm), methanol (3000 ppm), pyridine (200 ppm), toluene (890 ppm), N, N-dimethylformamide (880 ppm), and acetonitrile (410 ppm) are of Class II solvents whereas class III solvents are acetic acid, acetone, isopropyl alcohol, butanol, ethanol and ethyl acetate and they have been permitted daily exposures of 50 mg or less per day. In this regard, ICH guidelines for limits should be strictly followed.

2. Impurities related to formulation

Apart from bulk drug-related impurities, the formulated form of API may contain impurities that form in various ways.

a) Process related

b) Environmental related

c) Dosage form factors related

d) Mutual interaction amongst ingredients

e) Functional group- related

f) Degradation

Once a decision has been made to identify unknowns, the next logical step is to evaluate all known process related impurities, precursors, intermediates, and degradation products. By observing HPLC relative retention times (RRT) of all known process related impurities precursors and intermediates (if available), one can quickly determine whether or not the impurity of interest is truly unknown. If the relative retention time of the unknown impurity matches that of a standard then the unknown can be identified using HPLC with ultra-violet (UV) photodiode array as well as mass spectrometry (MS) detection. The identity is confirmed by correlating the retention time, UV spectra and mass spectra of the unknown impurity with that of the standard.

Identification of an unknown by using a standard, as described in the above paragraph, is a quick and easy process, but when the relative retention time of an unknown does not match with that of a standard than molecular mass and fragmentation data via HPLC-MS will be required. It is essential to determine the molecular mass of the unknown. Not only does the molecular mass help in the identification of the unknown, but it also enables one to track the correct peak by

HPLC if isolation becomes necessary. In order to run LC-MS, a mass spectrometry compatible HPLC method must be available. The mobile phase should contain volatile buffers that are HPLC-MS-compatible.

If the mass spectrometry data evaluation yields sufficient structural information, this eliminates the need to isolate the impurity in question. If standards of the proposed structures are available, they can be correlated with the unknown as previously described. If standards are not available, isolation is required.

An alternative to isolation is the small-scale synthesis. If possible structures have been proposed from the mass spectrometry data, one can study the process chemistry and determine at which step of the process the impurity and/or degradant is most likely to be formed. By knowing the process chemistry, the feasibility to synthesize the proposed impurity can be evaluated. The proposed impurity can then be synthesized if a reasonable synthetic route is available. It is easier to synthesize and identify the unknown if the chemistry works quickly (i.e. one step/straight-forward synthesis). If small-scale synthesis is chosen, the synthesis must be the most efficient route.

At this stage of the process, it is frequently necessary to isolate and characterize the unknown. One of the most important factors to consider when approaching an isolation experiment is the sample origin. It is vital to determine whether the unknown is an impurity and/or degradant, and to locate a sample that contains an enriched

quantity of the unknown. Isolating low level impurities can prove to be very cumbersome and time consuming. Therefore, the ultimate goal is to find a sample that contains an enriched quantity of the unknown. Two great resources of enriched samples are retained mother liquor samples and purposeful degradation/stability samples. If the unknown is a drug substance degradant, then the degradation reaction can be scaled-up to generate a large quantity of the unknown. If it is a drug product degradant, then effort should be put forth to form the degradant in the drug substance so that extraction from the excipients is not required. Whenever enriched samples are not available, the unknown must be isolated from the bulk drug substance or drug product.

A number of methods can be used for isolating impurities and/or degradants. Three of the most utilized techniques are preparative thin-layer chromatography, flash column chromatography and preparative high performance liquid chromatography. The actual technique used depends upon the nature of the impurity and/or degradant, including the amount present in the original material from which it must be isolated.

Complete isolation work have been carried out using final API in which impurities are present at 0.1 to 0.18% therefore preparative HPLC technique of isolation is a ideal choice.

Preparative HPLC technique

Preparative chromatography is the process of using liquid chromatography to isolate a sufficient amount of pure unknown compound(s) for the purpose of structure elucidation by various spectroscopic techniques, which are often referred to as semi-preparative.

The scale of preparative HPLC is normally larger than that of conventional HPLC (analytical HPLC). Therefore, a practical starting point is to develop a suitable analytical method. Optimization of the analytical method implies seeking conditions, which combine maximum resolution of the peak of interest and minimum elution time, under the restriction of a limited pressure drop. The optimized conditions determine the column, mobile phase, flow rate and sample loading capacity for the particular column. The conditions may be either normal phase or reverse phase. The mobile phase should be chosen carefully to avoid salt complexation with the compound to be isolated. Volatile acid salts such as trifluoroacetic acid, formic acid and acetic acid are acceptable mobile phase additives, and the ammonium counter-ion is preferred for pH adjustment to any of these acids.

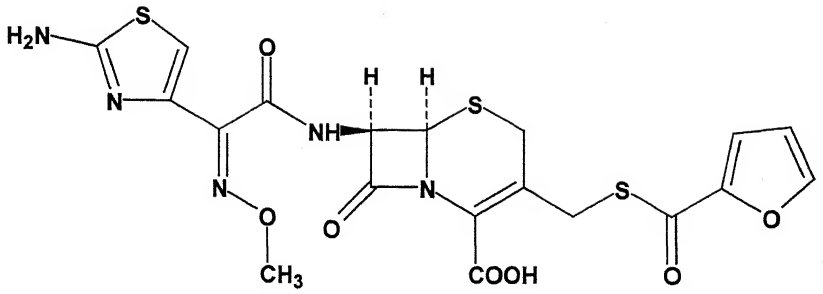
Once the analytical scale method conditions are optimized, the next step is to choose a semi-preparative column and scale-up the analytical HPLC parameters so that preparative chromatography can

be performed and the unknown compound(s) isolated for identification can be done by MS and NMR.

When the preparative method has been optimized, injections are made and the compound of interest is typically collected using a fraction collector. The fractions are pooled together in a collection vessel. The stability of the isolated product should be assessed prior to isolation in order to determine if special collection conditions are required. The isolated product is concentrated using distillation at normal or reduced pressure, freeze-drying & membrane filtration. After the product has been recovered, it should be dried under high vacuum to remove all solvents. An analytical clean up of the isolated sample is critical prior to NMR analysis. A clean sample improves the purity and quality of NMR data. It is essential to remove any salts and/or impurities from the isolated product. A simple purification method for the isolated product is to re-inject it onto the preparative column using a mobile phase without any additives or pH adjustments. By utilizing gradient elution, salts can be removed by incorporating an aqueous rinse at the beginning of the run, and then the organic solvent can be ramped to elute the desired product. Thus, the isolated peak is purified and submitted for LC-MS and NMR analysis.

Suitable HPLC methods have been developed to separate impurities present in ceftiofur, cefuroxime axetil & cefprozil.

CEFTIOFUR



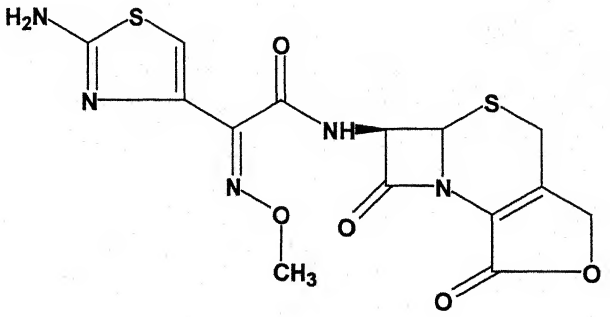
Ceftiofur

Impurities

- Cefotaxime lactone
- Desacetyl Cefotaxime
- Ceftiofur Thiol
- Disulfide of Ceftiofur
- Disulfide of thiofuric acid

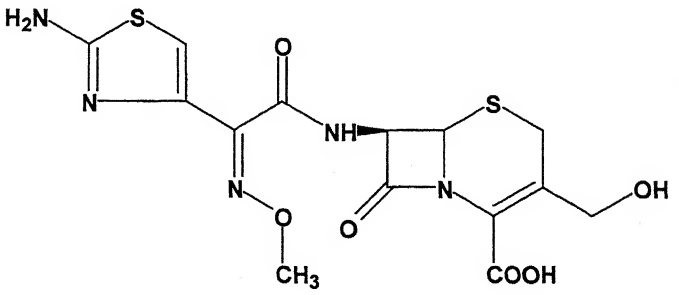
Cefotaxime lactone

N-((2R)-1,7-dioxo(5-hydro-2H,4H,2aH-azetidino[2,1-b]furano [3,4-d]1,3-thiazin-2-yl))(2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza -3-methoxyprop-2-enamide



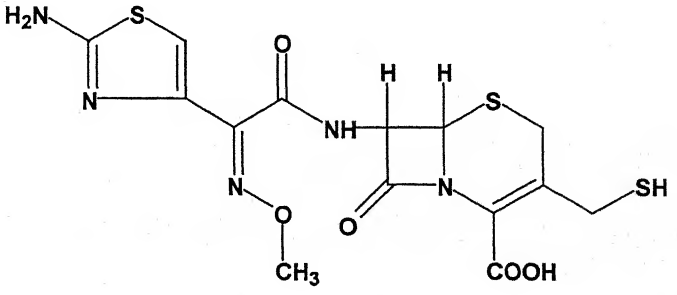
Desacetyl Cefotaxime

(6R)-6-[(2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza-3-methoxyprop-2-enoylamino]-3-(hydroxymethyl)-5-oxo-2H,6H,6aH-azetidino[2,1-b]1,3-thiazine-4-carboxylic acid



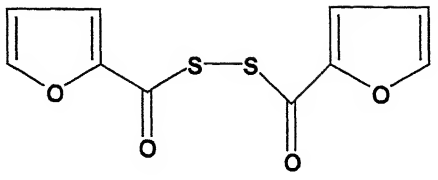
Ceftiofur thiol

6-[(2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza-3-methoxyprop-2-enoylamino]-5-oxo-3-(sulfanylmethyl)-2H,6H,6aH-azetidino[2,1-b]1,3-thiazine-4-carboxylic acid



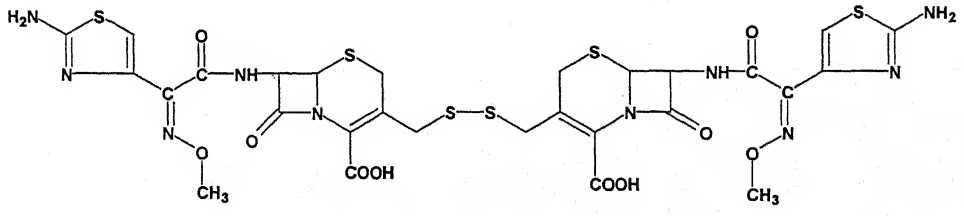
Disulfide of thiofuric acid

2-furyl (2-furylcarbonyl)disulfanyl ketone



Disulfide of ceftiofur

6-[(2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza-3-methoxyprop-2-enoylamino]-3-{[({6-[(2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza-3-methoxyprop-2-enoylamino]-4-carboxy-5-oxo(2H,6H,6aH-azetidino[2,1-b]1,3-thiazin-3-yl)}methyl)disulfanyl]methyl}-5-oxo-2H,6H,6aH-azetidino[2,1-b]1,3-thiazine-4-carboxylic acid



Chromatographic conditions :

Column

- Type: X-Terra
- Dimensions: 150m x 3.9 mm
- Particle size: 5μm

Detector setting

- Wavelength: 235nm

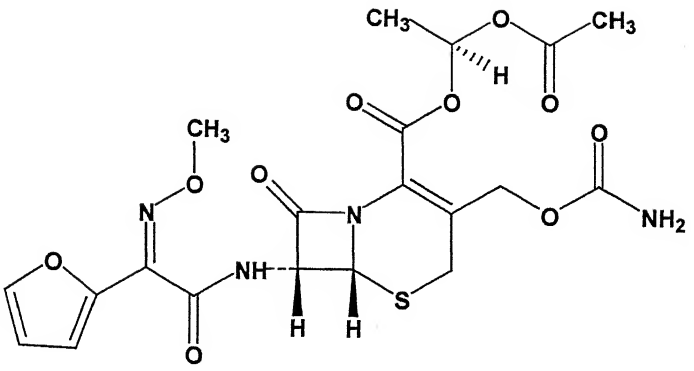
Mobile phase

- Buffer: 0.01M Ammonium Di hydrogen Orthophosphate + 0.07 % Tetra heptyl ammonium bromide (THAB.)
- Mobile A: B Buffer: Methanol (62:38 v/v)
- pH: 6.5 with dilute Ammonia (10%)
- Flow rate: 1.80ml/min

Gradient Program

S.No.	Time	Event	Value	Flow ml/min
1.	0.01	B.conc	0.00	15.0
2.	5.00	B.conc	0.00	15.0
3.	10.00	B.conc	15.00	15.0
4.	20.00	B.conc	50.00	15.0
5.	25.00	B.conc	50.00	15.0
6.	30.00	B.conc	60.00	15.0
7.	35.00	B.conc	0.000	15.0

CEFUROXIME AXETIL



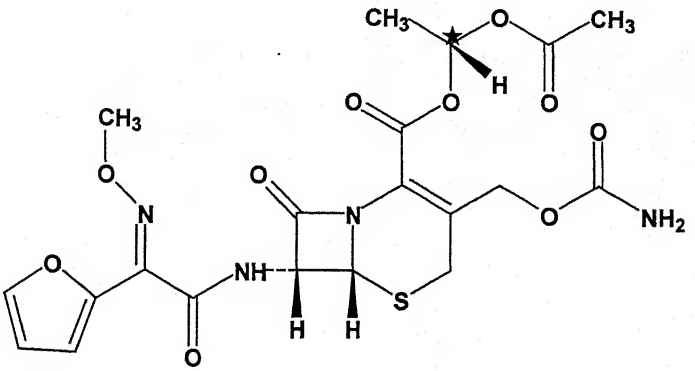
Cefuroxime Axetil

Impurities

- Anti isomer - A
- Anti isomer – B
- Delta Isomer

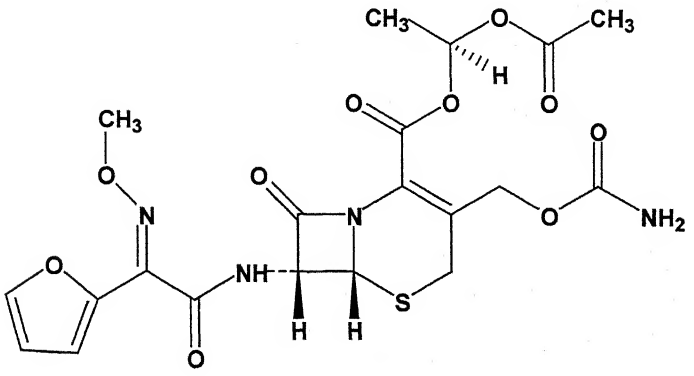
Anti Isomer –A

{6-((2E)-3-aza-2-(2-furyl)-3-methoxyprop-2-enoylamino)(6R)-3-[(aminocarbonyloxy)methyl]-5-oxo-2H,6H,6aH-azetidino[2,1-b] 1,3-thiazin-4-ylcarbonyloxy}ethyl acetate.
(Anti isomer-A of Cefuroxime Axetil)



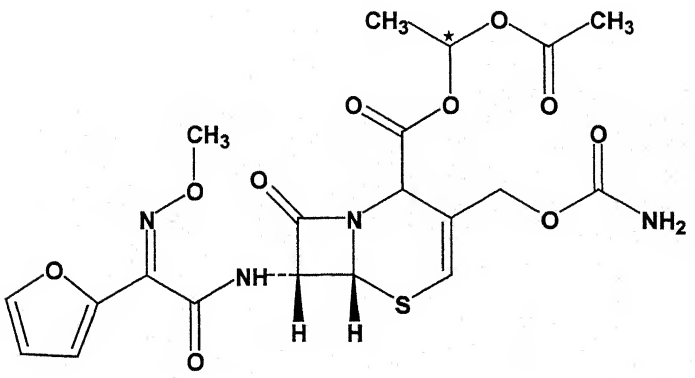
Anti Isomer –B

{6-((2E)-3-aza-2-(2-furyl)-3-methoxyprop-2-enoylamino)(6R)-3-[(aminocarbonyloxy)methyl]-5-oxo-2H,6H,6aH-azetidino[2,1-b] 1,3-thiazin-4-ylcarbonyloxy}ethyl acetate.
(Anti-B isomer of Cefuroxime axetil)



Delta Isomer

{(6R)-6-((2Z)-3-aza-2-(2-furyl)-3-methoxyprop-2-enoylamino)-3-[(aminocarbonyloxy)methyl]-5-oxo-4H,6H,6aH-azetidino[2,1-b] 1,3-thiazin-4-ylcarbonyloxy}ethyl acetate.
(Delta-2 isomer of cefuroxime axetil)



Chromatographic conditions :

Column

- Type: Zorbax SB Phenyl
- Dimensions: 250 mm x 4.6 mm
- Particle size: 5µm

Detector setting

- Wavelength: 280 nm

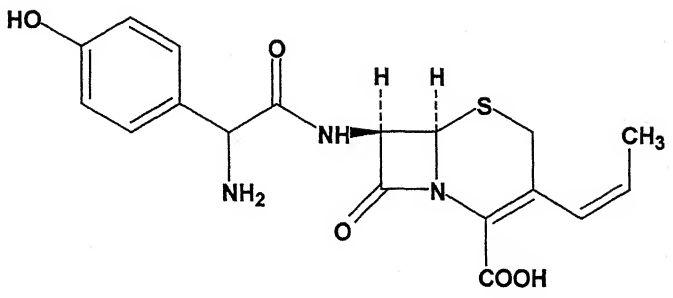
Mobile phase

- Buffer: 02.5317 g KH2PO4 + 9.8013 g Tetrabutyl Ammonium Hydrogen Sulphate dissolved in 1400 ml water.
- Mobile A: 775 ml Buffer + 163 ml Acetonitrile + 42 ml THF.
- Mobile B: 600 ml Buffer + 326 ml Acetonitrile + 74 ml THF.
- PH: 2.42 with Phosphoric acid
- Flow rate: 1.30ml/min

Gradient Program

S.No.	Time	Event	Value	Flow ml/min
1	0.01	B.conc	0.00	1.3
2	14.00	B.conc	0.00	1.3
3	30.00	B.conc	40.00	1.3
4	55.00	B.conc	40.00	1.3
5	60.00	B.conc	0.00	1.3
6	65.00	B.conc	0.00	1.3

CEFPROZIL



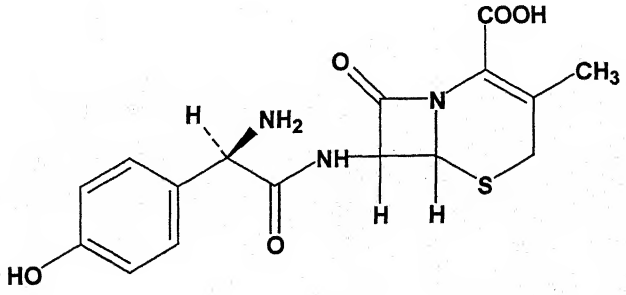
Cefprozil

Impurities

- Cefadroxil
- PHPG Cefprozil (p-hydroxy phenyl glycine cefprozil)
- Cefprozil Carbonate

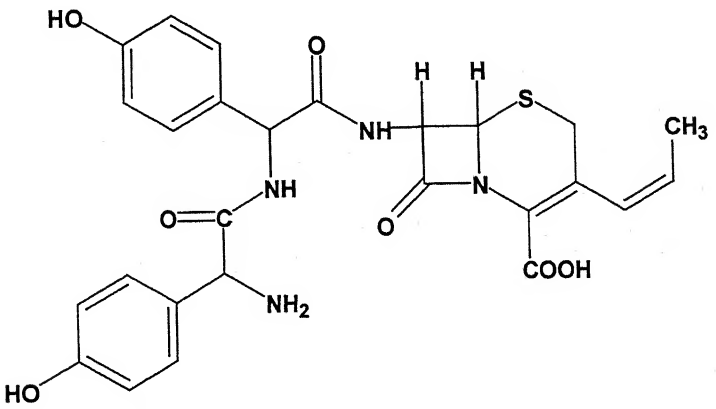
Cefadroxil

(6R, 7R)-7-[[(2R)-2-Amino-2- (4-hydroxyphenyl) acetyl] amino]-3-methyl-8-oxo-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid.



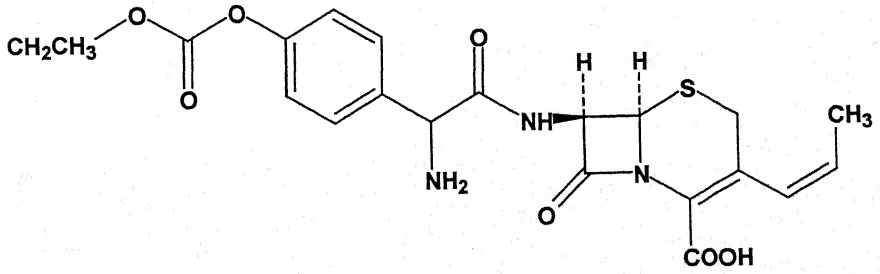
PHPG Cefprozil

3-((1Z)prop-1-enyl)-6-{2-[2-amino-2-(4-hydroxyphenyl)acetylamino]
-2-(4-hydroxyphenyl)acetylamino}-5-oxo-2H,6H,6aH-azetidino
[2,1-b]1,3-thiazine-4-carboxylic acid. (PHPG Cefprozil)



Cefprozil Carbonate

(6R)-3-((1Z)prop-1-enyl)-6-[2-amino-2-(4-ethoxycarbonyloxyphenyl)
acetylamino]-5-oxo-2H,6H,6aH-azetidino[2,1-b]1,3-thiazine-4-
carboxylic acid.(Cefprozil Carbonate)



Chromatographic conditions:

Column

- Type: Inertsil C_{18}
- Dimensions: 250mm x 4.6 mm
- Particle size: 5µm

Detector setting

- Wavelength: 280nm

Mobile phase

- Buffer: 0.01 M Sodium Dihydrogen Orthophosphate
- Mobile A: Buffer: ACN (90: 10 v/v)
- Mobile B: Buffer: ACN (30: 70 v/v)
- pH: 4.4 with Orthophosphoric Acid
- Flow rate: 1.5 ml/min

The solutions were filtered and each mobile phase was de-aerated separately.

Gradient Program

S.No.	Time	Event	Value	Flow ml/min
1.	0.01	B.conc	0.00	1.5
2.	15.00	B.conc	5.00	1.5
3.	35.00	B.conc	25.00	1.5
4.	70.00	B.conc	50.00	1.5
5.	72.00	B.conc	0.00	1.5

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